THE PHYSIOLOGY, BIOCHEMISTRY AND GENETICS OF PROPANE METABOLISM IN RHODOCOCCUS RHODOCHROUS PNKB1

BY

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Dedication

To my father who made it all possible;
Amel, who showed me the path
and kept me on it.

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Declaration

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. J.C. Murrell. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

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Summary

The metabolism of propane by R.rhodochrous PNKb1 has been investigated using various techniques. SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane and some of the potential oxidation intermediates in the metabolism of propane showed the synthesis of three specific polypeptides of approximately 69, 59 and 57 kDa. A putative propane oxygenase activity was detected in cell-free extracts only by the formation of 1,2-epoxypropane from propene. Propane was shown to be a competitive inhibitor of propene oxidation activity.

Activity of the putative propane oxygenase was only observed in propane and acetol-grown cells. The effect of various inhibitors on the formation of 1,2-epoxypropane from propene in whole cells of propane and acetol-grown R.rhodochrous PNKb1 was investigated. This showed that the epoxide formation was inhibited by the same inhibitors under both conditions, suggesting a relationship between the metabolism of propane and acetol. Acetol monooxygenase (AMO) activity was also detected in acetol-grown cells. This enzyme was partially purified using gel filtration and ion-exchange chromatography. However, neither NAD^+ nor $NADP^+$-dependent acetol dehydrogenase activity was measured in cell-free extracts of acetol-grown cells, indicating that acetol is not metabolized via a pyruvate pathway. NAD^+-dependent secondary alcohol dehydrogenase activity in cell-free extracts of propane and propan-2-ol-grown cells was shown to be higher than the primary alcohol dehydrogenase. 1,2-Propanediol dehydrogenase activity in 1,2-propanediol-grown cells was six-fold higher than the corresponding activity obtained in propane-grown cells, indicating that there is more than one alcohol dehydrogenase involved in the metabolism of propane. NAD^+-dependent secondary alcohol dehydrogenase was synthesized in cell-free extracts of propane grown-cells. This enzyme was also synthesized after growth on some of the potential intermediates in the subterminal oxidation pathway including 1,2-propanediol.

NTG-generated mutants blocked in certain steps of the propane oxidation pathway were used to study the terminal and subterminal oxidation of propane metabolism. Mutants defective in propane metabolism (alk$^-$) were also unable to grow on acetol, but still grew on terminal and subterminal intermediates of propane oxidation. SDS-PAGE analysis of alk$^-$ mutants grown under propane-inducing condition showed the synthesis of at least one of the propane-specific polypeptides. This indicated that those mutants are defective in a regulatory gene(s) (alkR) of propane/acetol oxygenase.

Culture supernatants during growth on propane and potential intermediates of propane metabolism were analysed. This showed that methyl acetate is not an intermediate in the metabolism of propane.

Ultra-thin sections of propane-grown R.rhodochrous PNKb1 showed the involvement of unusual intracellular structures at the poles of the cells. These structures could be associated with propane assimilation.

Abbreviation

AMO	Acetol monooxygenase
AMS	Ammonium mineral salts medium
CMN	Corynebacterium-Mycobacterium-Nocardia complex
CoA	Coenzyme A
CW	Cell wall
DEB	1,2,3,4 Di-epoxybutane
DEO	1,2,7,8 Di-epoxyoctane
EDI	Electron-dense inclusions
EDTA	Ethylene diamine tetraacetic acid
FID	Flame ionization detector
FPLC	Fast performance liquid chromatography
G.C	Gas chromatography
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
kDa	Kilodalton
K_m	Michaelis constant
MDH	Methanol dehydrogenase
MMO	Methane monooxygenase
MPA	Meat-peptone agar
MPa	Mega pascal
$NAD(P)^+$	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
NTG	N-methyl-N-nitrosoguanidine
O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PMO	Propane monooxygenase

1,2-PDL	1,2-Propanediol
PQQ	Pyrroloquinoline quinone
RDI	Round dense inclusions
rpm	Revolutions per minute
S	Septum
SDS	Sodium dodecyl sulphate
Str^r_{20}	Streptomycin resistant at a concentration of 20 $\mu g\ ml^{-1}$
$T_{1/2}$	Half life
TZ	Transparence zone
TRIS	Tris (hydroxymethyl) -methylamine
V_{max}	Maximal possible reaction velocity
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

CHAPTER 1

INTRODUCTION

1.1 The Rhodococcus genus

Extensive studies of the biology and genetics of the genus Rhodococcus have been reviewed by Finnerty, 1992. Species from the genus Rhodococcus were previously assigned to genera such as Arthrobacter, Brevibacterium, Corynebacterium, Mycobacterium, and Nocardia (Goodfellow and Cross, 1984). The genus Gordona and Tsukamurella were also considered as rhodococci (Goodfellow *et al.*, 1991). The genus Rhodococcus consists of thirteen species according to the current edition of Bergey's Manual of Systematic Bacteriology (Goodfellow, 1989).

The rhodococci are aerobic, Gram positive, catalase positive and non-motile. The morphogenetic cycle is initiated with cocci, which germinate to produce short rods. These then form filaments with elementary branching. Some members of the genus Rhodococcus are pathogenic for humans, animals and plants. Rhodococci are recognised by their ability to degrade toxic compounds (as the sole source of carbon and energy) such as polycyclic aromatic hydrocarbons (PAHs) including naphthalene, anthracene and fluoranthene (Gibson, 1984; Cerniglia, 1984; Walter *et al.*, 1991). They also exhibit a broad substrate diversity for the degradation of halogenated alkanes, anilines, phenols and halogenated phenols. Rhodococci are also used in many industrial applications, for example, the production of acrylic acid and acrylamide and the interconversion of steroids (Cited by Finnerty, 1992). Nitrilases and nitrile hydratases from

rhodococci are commonly used for many industrial purposes. These enzymes are responsible for the transformation of aliphatic and aromatic nitriles to acids and amides, respectively (Nagasawa *et al.*, 1990; Nagasawa *et al.*, 1991).

1.2 The microbial metabolism of alkanes

1.2.1 Historical perspective

Owing to the potential use of petroleum hydrocarbons as substrates by the fermentation industry (Vestal, 1984), the metabolism of hydrocarbons received great attention from researchers in the 1950s and 1960s . With the increased cost of petroleum in the 1970s, fundamental studies investigating the biochemistry of oxygenase enzymes began (e.g. Cardini and Jurtshuk, 1970; Colby and Dalton, 1976). Particular interest has been directed in the 1980s at understanding and developing genetics and molecular biology of hydrocarbon utilizers (e.g. Owen *et al.*, 1984).

Microorganisms that utilize hydrocarbons, as a source of carbon and energy, are known as hydrocarbon utilizers. In 1961, Fuhs listed several microorganisms that had been reported as hydrocarbon utilizers, some 50% of which were capable of growth on long chain normal paraffinic hydrocarbons (C_{10} through C_{18}). The rest were able to utilize gaseous hydrocarbons (ethane, propane and butane) (Fuhs, 1961). Yet, compared with methane, the metabolism of higher gaseous alkanes (ethane, propane and butane) has received little attention in the literature. However, the

pathways for the metabolism of methane and liquid n-alkanes were studied in more detail since microorganisms had been used for the degradation of oil spills in the environment (Gutnick and Rosenberg, 1977).

"Any study of higher gaseous alkane metabolism cannot be considered in isolation from methane or liquid alkane metabolism about which relatively more is known" (Ashraf, 1990). Therefore, a brief historical background of the physiology and biochemistry of methane and liquid n-alkane is summarized here (for more reviews see Watkinson, 1980; Perry, 1980; Stephens, 1983; Britton 1984; Singer and Finnerty, 1984a; Vestal, 1984; Watkinson and Morgan, 1990).

1.2.2 Methane-oxidizing bacteria

Methylotrophs (C_1-utilizers) are recognised by their ability to reduce one-carbon compounds such as methane, methanol, N-methylamine and methylated sulfur, as the sole source of carbon and energy, and to assimilate formaldehyde derived from the primary substrate for the synthesis of cell material (Anthony, 1982 and Zatman, 1981). In 1892, Leow isolated the first methylotrophic bacterium that grew on methanol, methylamine , formic acid and several multicarbon compounds. This organism was designated Bacillus methylicus (see Quayle, 1987). The traditional definition of methylotrophs given above was by Colby and Zatman (1973). However, microorganisms that utilize methane (and methanol) are known as methanotrophs. The first methanotrophic

bacterium was isolated by Söhngen in 1906, and named Bacillus methanica. In 1909, the name was changed to Methanomonas methanica by Orla-Jensen, to Pseudomonas methanica by Dworkin and Foster and finally to Methylomonas methanica by Foster and Davis (see Hanson, 1992).

Stephens (1983) surveys much of the evidence against the existence of facultative methane-oxidizers and suggests reasons why obligate methanotrophs exist. The evidence rests on two points, namely the purity of the substrates employed for growth studies and the purity of culture itself.

In 1970, over 100 methane utilizing bacteria were isolated by Whittenbury and his colleagues. They divided them into five groups (Whittenbury *et al.*, 1970). Methanotrophs were then divided into three types (I, II and X) based generally on the internal cytoplasmic membrane arrangement, cell shape and carbon assimilation pathways (for more details see Hanson, 1992). Type I and type X methanotrophs assimilate formaldehyde via a ribulose monophosphate (RuMP) pathway, while type II and facultative methylotrophs use the serine pathway (Lawrence and Quayle, 1970). Type I methanotrophs have membranes as bundles of vesicular disks, whilst Type II organisms have paired membranes around the periphery of the cell. Obligate and facultative methylotrophs are unable to utilize methane and do not possess complex intracytoplasmic membrane structures (Anthony, 1982).

It has been shown that methane is oxidized to methanol. This is further metabolized to formaldehyde, then to formate and finally to carbon dioxide (Anthony, 1982; Dalton and Leak, 1985; Anthony, 1986).

Methane monooxygenase (MMO) is the enzyme responsible for the oxidation of methane to methanol in methanotrophic bacteria (Colby and Dalton, 1976;1978). MMO is found as either a particulate (membrane-bound) or a soluble (cytoplasmic) protein (Stanley *et al.*, 1983). Soluble MMO of Methylococcus capsulatus (Bath) was extensively characterized by Colby and Dalton, 1976; Green and Dalton, 1985; Lund *et al.*, 1985. This enzyme consists of three components (A, B and C). Protein A consists of three subunits, α, β and γ (Green and Dalton, 1985). Particulate MMOs were found in Methylomonas methanica and Methylosinus trichosporium OB3b (Colby *et al.*, 1975 and Tonge *et al.*, 1975,1977).

Methanol dehydrogenase (MDH) from both methane- and methanol- utilizing bacteria has received special attention in the literature (for more details see Colby *et al.*, 1979). Most studies were focused on the enzyme from Gram-negative organisms. Anthony reviewed the presence of a broad specificity NAD(P)-independent methanol dehydrogenase from both methane and methanol utilizing bacteria (Anthony, 1986). It has been proved that methanol dehydrogenase contains a pyrrolo-quinoline quinone (PQQ) prosthetic group (Duine and Frank, 1980).

Formaldehyde can be either assimilated to biomass or oxidized to CO_2. Formaldehyde dehydrogenase is involved in the latter pathway of which there are two types; a $NAD(P)^+$-independent and a $NAD(P)^+$-dependent. An $NAD(P)^+$-dependent formaldehyde dehydrogenase was purified from Methylococcus capsulatus (Bath) (Stirling and Dalton, 1978).

A considerable amount of information on the genetic analysis of C_1 utilizing microorganisms has accumulated steadily (for reviews see Lidstrom-O'Connor, 1983; Lidstrom *et al.*, 1987; Holloway, 1984 and Holloway *et al.*, 1987). The genetics and molecular biology of obligate methane-oxidizing bacteria has been extensively discussed by Murrell (1992).

For more review of C_1-utilizers see Crawford and Hanson, 1984; Atlas, 1984; Andreesen and Bowien, 1989; Goldberg and Rokhem, 1991; Murrell and Dalton, 1992; Murrell and Kelly, 1993).

1.2.3 The metabolism of liquid n-alkanes

1.2.3.1 Historical perspective

Crude oil is the most obvious source of liquid n-alkanes (C_5-C_{15}). These alkanes are widely spread in the environment, as a result of human activity, plant and microbial production. The total input of petroleum from all sources into the sea in 1985 was estimated by the National Research Council as approximates 8.9×10^8 US gallons (1 US

gallon = 0.83 Imperial gallon, 3.8 litres) (cited by Prince, 1992).

Tornabene (1976) listed various microorganisms that utilize hydrocarbons and long chain n-alkanes (C_{15}-C_{35}). More recently, Rosenberg and Gutnick (1981) listed the following genera as being the most frequently isolated from hydrocarbon enrichments: Pseudomonas, Acinetobacter, Flavobacterium, Brevibacterium, Corynebacterium, Arthrobacter, Mycobacterium and Nocardia.

Watkinson and Morgan (1990) reviewed many aspects of the physiology, biochemistry and genetics of alkanes larger than methane, alkenes and alkynes emphasising the recent developments.

Gaseous alkanes (methane, ethane, propane and butane) are somewhat soluble in water. They have an aqueous concentration of 0.2-2 mM (Watkinson, 1980). This is enough to support observed growth rates. Solubility of liquid n-alkanes from hexane to hexadecane falls from 0.1 mM to 0.03 μM (Bell, 1973). However, Stephens (1983) discussed most of the possible mechanisms for the initial interaction of substrates that have low solubility in water such as liquid n-alkanes with microbial cells. She also explained why n-alkane utilization is so common in CMN-complex bacteria, see section 1.3 (for more details see Watkinson, 1980; Stephens, 1983 and Britton, 1984).

1.2.3.2 Pathways of liquid n-alkane utilizers

a) Terminal oxidation

It has been suggested by several groups of investigators that both short- and long-chain alkanes are oxidized monoterminally to the corresponding primary alcohol, aldehyde and then to fatty acids (Mckenna and Kallio, 1965). Fatty acids derived from alkanes are then oxidized through a ß-oxidation system to the level of acetate (or propionate for odd chain length n-alkanes) (Fig. 1.1).

The wax ester, cetyl palmitate, accumulated in a culture of Acinetobacter sp. HO1-N during growth on hexadecane (Stewart *et al.*, 1959). Hydroperoxides are potential intermediates in alkane oxidation in Acinetobacter sp. HO1-N due to the formation of esters from alkyl hydroperoxide-grown cells and the rapid oxidation of n-alkyl hydroperoxides. A proposed pathway is shown in Fig. 1.1 (Stewart *et al.*, 1959).

However, dicarboxylic acids accumulated in the culture of Corynebacterium sp. after growth on (C_{10}-C_{14}) n-alkanes (Kester and Foster, 1963), see Fig. 1.1.

Much of the evidence for a terminal oxidation pathway was based on the detection of the homologous fatty acids in the culture media indicating that terminal oxidation is the most common pathway of alkane degradation.

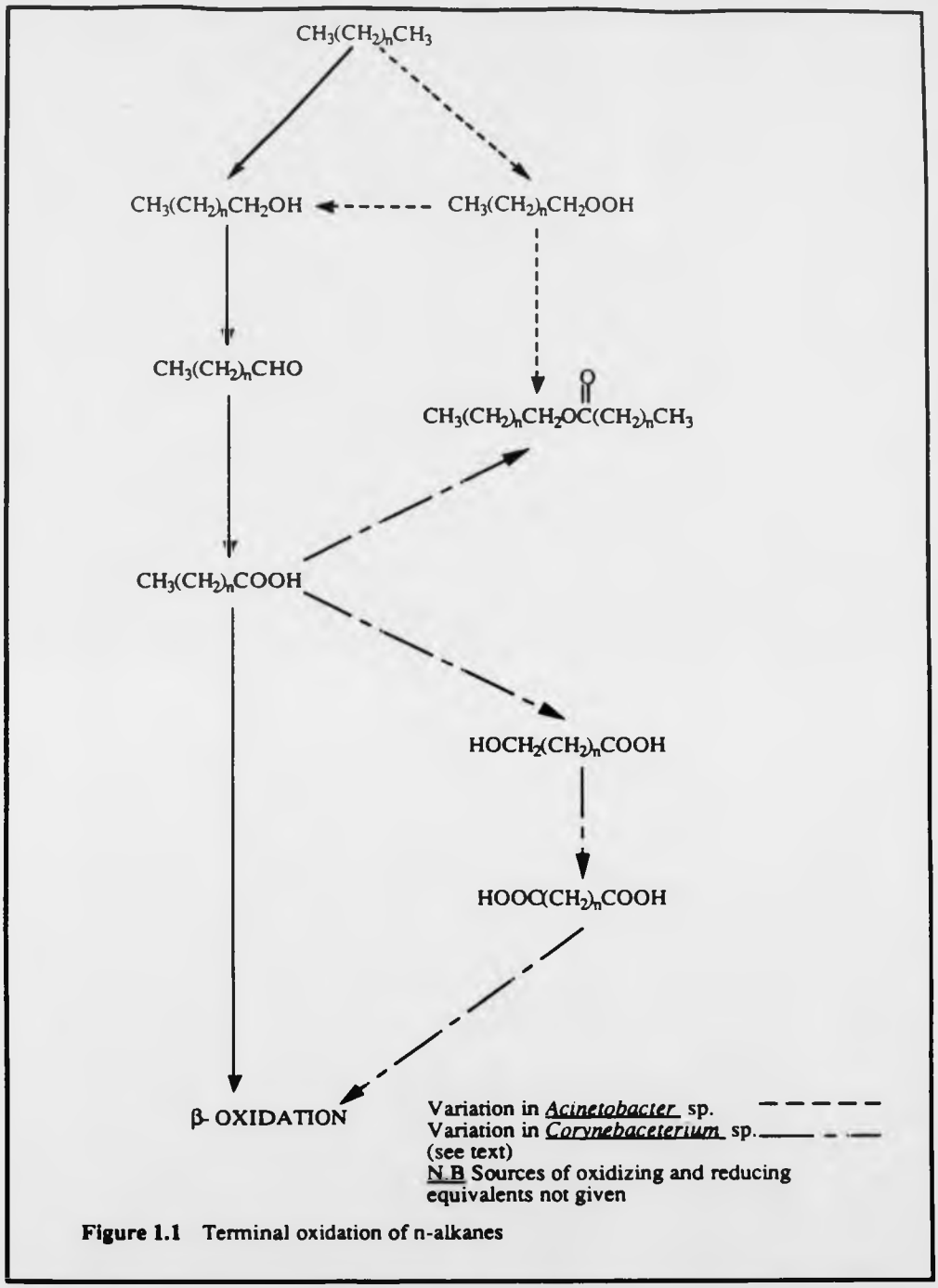

Figure 1.1 Terminal oxidation of n-alkanes

Ratledge (1978) and Rehm and Reiff (1981) reported an extensive list of microorganisms that have the ability to convert alkanes to dicarboxylic acids via di-terminal oxidation (Fig. 1.2). Recently, Blasing *et al.* (1988,1989) described both mono- and di-terminal oxidation systems in Candida sp. In the diterminal pathway, alkane is oxidized by the typical monoterminal route to the corresponding fatty acids. Subsequently, monocarboxylic fatty acid is oxidized to ω-hydroxy acid, ω-aldehyde fatty acid and then to a dicarboxylic fatty acid. Dicarboxylic acids have accumulated in the culture after growth on n-alkanes of several organisms such as Candida (Yi and Rehm, 1982a&b) and Corynebacterium (Kester and Foster, 1963). There is also weak evidence that n-alkane was metabolised via alkenes. This pathway was described as being relatively slow (Ratledge, 1978 and Singer and Finnerty, 1984a). Terminal and di-terminal oxidation pathways were extensively discussed by Singer and Finnerty (1984a) and Buhler and Schindler (1984).

b) Subterminal oxidation

Subterminal alkane oxidation pathways have been reviewed by Markovetz (1971) and Stephens (1983). The first intermediate in the subterminal oxidation pathway is a secondary alcohol, which is subsequently oxidized to a ketone. Secondary alcohols and methyl ketones were formed preferentially during co-oxidiation of C_{10}-C_{16} n-alkanes by Arthrobacter sp. (Klein *et al.*, 1968). The further step is the formation

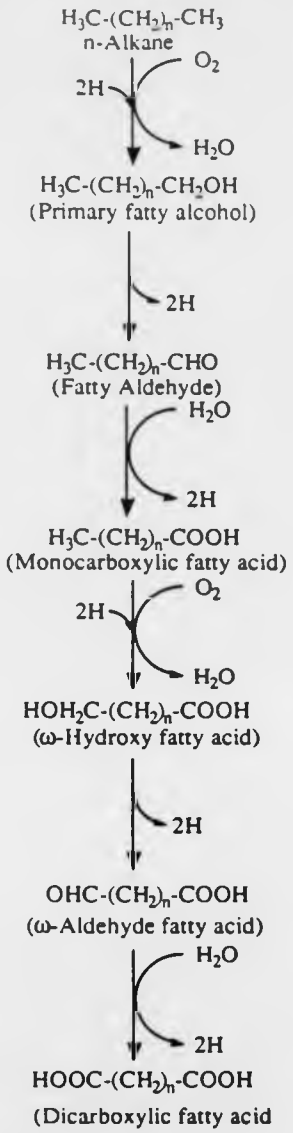

Figure 1.2 Pathway of di-terminal alkane oxidation (Adapted from Rehm and Reiff, 1981).

of ester from ketones. Markovetz and his colleagues studied the metabolism of tridecan-2-one by several Pseudomonas species. They suggested that tridecan-2-one was oxidized to undecyl acetate. This was then followed by hydrolysis to undecan-1-ol and acetate (Forney and Markovetz, 1968), and subsequently to undecanoic acid (Fig. 1.3).

1.2.3.3 Anaerobic oxidation of n-alkanes

There is some evidence for the anaerobic degradation of alkanes. This involves the dehydrogenation of an n-alkane to alkene, which subsequently is hydroxylated to primary alcohols. Most of the convincing evidence has come from Schink (1985a).

Under anaerobic conditions, NAD-dependent alkane dehydrogenase and NADH-dependent alkene hydroxylase were partially purified from a hexadecane-grown Pseudomonas sp. Decane was converted to dec-1-ene by the dehydrogenase and then from decene to the corresponding alcohol by a hydroxylase (Parekh et al., 1977).

Schink (1985b) reported a new species that grew anaerobically on ethyne. This was hydrated to acetaldehyde and then to acetate and ethanol.

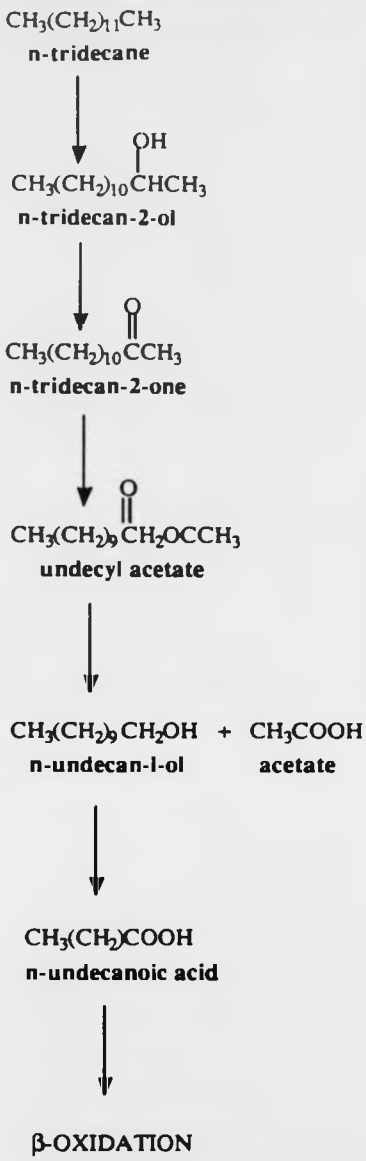

Figure 1.3 Pathway of sub-terminal oxidation of n-tridecane in *Pseudomonas* sp. (adapted from Klug & Markovetz, 1971)

N.B Sources of oxidizing and reducing equivalents not given

1.2.3.4 Mechanisms of n-alkane oxidation

Three mechanisms have been proposed for the oxidation of n-alkanes to their corresponding primary alcohols. These mechanisms are summarized as follows:

(1) $RCH_2CH_3 + O_2 \rightarrow RCH_2CH_2OOH + NAD(P)H + H^+ \rightarrow RCH_2CH_2OH + NAD(P)^+ + H_2O$

(2) $RCH_2CH_3 + NAD(P)^+ \rightarrow RCH{=}CH_2 + NAD(P)H + H^+ + H_2O \rightarrow RCH_2CH_2OH$

(3) $RCH_2CH_3 + O_2 + NAD(P)H + H^+ \rightarrow RCH_2CH_2OH + NAD(P)^+ + H_2O$

Indirect evidence for the formation of alkyl hydroperoxides was given by Stewart *et al.* (1959) (equation 1). They showed that alkyl hydroperoxides were oxidized by Acinetobacter HO1-N after growth on hexadecane. Cetyl palmitate was formed in the metabolism of alkyl hydroperoxide (Finnerty *et al.*, 1962). Alkyl hydroperoxides were also degraded in cell-free extracts of Acinetobacter sp. suggesting that an NADH-dependent alkyl hydroperoxide reductase was present in this organism (McKenna and Kallio, 1965). Recently, an alkyl hydroperoxide reductase was purified from Salmonella typhimurium (Jacobson *et al.*, 1989). Studies using Pseudomonas olevorans showed that rubredoxin can act as an electron carrier in the reduction of alkyl hydroperoxide to the corresponding alcohols (Boyer *et al.*, 1971).

The dehydration reaction (equation 2) was suggested by Senz and Azoulay (1961). They detected hept-1-ene in a culture of heptane-grown Pseudomonas aeruginosa. Alkenes could be then hydroxylated to primary alcohols or oxygenated to the epoxide which could be reduced to alcohols. However, there are many arguments against this mechanism (see Mckenna and Kallio, 1965; Klug and Markovetz, 1971).

Equation 3 describes the most accepted mechanism for alkane oxidation. A mixed-function oxygenase (monooxygenase) system catalyzes the incorporation of one atom of molecular oxygen into the alkane molecule. However, dioxygenase systems have also been reported but these are less common. Equation 1 and 3 have the same overall stoichiometry and would both give the same results from $^{18}O_2$ labelling studies. Klug and Markovetz (1971) suggested that in the hydroperoxidation mechanism NAD(P)H would have the additional function of donating electrons to make the active oxygen species necessary for the formation of the alkyl hydroperoxide. They stated that this is contrary to the 1:1:1 ratio of O_2:NADH:octane observed with octane monooxygenase by Peterson *et al.* (1969) since NADH would be required to donate electrons for the formation of an active oxygen species and for the reduction of hydroperoxide to alcohol.

1.3 The metabolism of C_2-C_4 gaseous n-alkanes

Gaseous alkanes are widespread in nature. These compounds are metabolized by a variety of microorganisms which can be

isolated from soil or water samples using enrichment techniques. Most of these organisms are yeasts, filamentous fungi and bacteria. The latter are well studied, particularly the Corynebacterium-Mycobacterium-Nocardia (CMN) group and other genera such as Rhodococcus, Brevibacterium and Arthrobacter (for reviews see Foster, 1962; Perry, 1980 and Vestal, 1984).

It is a fact that hydrocarbons are relatively soluble in water. However, there is a controversy about in which form the hydrocarbons are utilized. This was raised by Britton (1984); Stephens and Dalton (1987); Watkinson and Morgan (1990). Although their argument was based on liquid hydrocarbon utilizers, some points may be pertinent to gaseous hydrocarbon utilizers. Firstly, cells can obtain substrate by adhering to submicron or large hydrocarbon droplets by cell-surface components. Secondly, cells produce surfactants to aid the dispersion of hydrophobic substrates. Studies of the outer layers of the cell wall in CMN-complex bacteria show the presence of mycolic acids, nocardols and nocardones . These make the cell surface extremely hydrophobic and facilitate the diffusion of a hydrophobic substrate into the organism. Finally, the presence of lipids and microbodies in hydrocarbon-grown cells suggests that there might be an active transport system in these microorganisms.

1.3.1 Pathways of C_2-C_4 gaseous n-alkanes

In comparison with ethane, propane and butane, the metabolism of methane and liquid n-alkanes have been widely investigated in the literature.
As described in section 1.2.3, liquid n-alkanes are metabolized *via* terminal and/or subterminal oxidations. It has been found that methane and liquid n-alkane-utilizing bacteria are capable of oxidizing n-alkanes *via* terminal and/or subterminal pathways (Colby *et al.*, 1977; Klein *et al.*, 1968 and Hou *et al.*, 1981). It might be that terminal and/or subterminal oxidations are involved in the metabolism of C_2-C_4 alkanes.

A brief historical background of ethane- and butane-utilizers is presented next, followed by a thorough discussion of propane utilizers.

1.3.2 Ethane

The metabolism of ethane has received scant attention essentially because it is such a small symmetrical molecule. It has been assumed that ethane is metabolized by a monooxygenase to ethanol, then to acetaldehyde and finally to acetate (for reviews see deBont and Albers, 1976; Vestal, 1984).

An unusual *Mycobacterium paraffinicum* strain was isolated by Davis *et al.* (1956). This species did not grow on ordinary

bacteriological media unless ethane was also supplied. It grows on ethanol, acetate and C_3-C_{10} n-alkanes. The authors suggest that ethene is an intermediate in the pathway of ethane oxidation, but this is unlikely because of its slow oxidation rate. In 1958, Dworkin and Foster isolated several ethane-utilizing Mycobacteria. However, attempts to study the possible metabolic scheme for ethane were unsuccessful due to the failures in obtaining ethane oxidation activity in cell-free extracts. de Bont and Albers (1976) found rather high levels of acetaldehyde dehydrogenase in ethane and ethanol-grown cells, but not in succinate or glucose-grown cells.

The metabolism of two-carbon gaseous hydrocarbons also includes ethylene and acetylene. For more details refer to Vestal (1984).

1.3.3 Propane

1.3.3.1 Introduction

Propane is widespread in petroleum deposits and represents 1-2% of natural gas. The advantage of employing propane as a substrate in the industrial fermentation processes lies in its low cost and its reasonable solubility in water.

The first propane-utilizing bacterium was described by Tausz and Donath in 1930 (cited by Perry, 1980). Most of the propane-utilizing bacteria belong mainly to CMN-complex and

other genera such as Brevibacterium. Rhodococcus and Arthrobacter (see Perry, 1980). Nearly all the research on propane metabolism has been done on Gram-positive bacteria. Takahashi *et al.* (1980) isolated one well documented Gram-negative propane-utilizing bacterium designated as Pseudomonas butanovora. This isolate is recognised by its ability to grow on n-alkanes from C_2-C_9. However, Stephens (1983) and Woods (1988) only observed Gram-positive organisms when isolating propane-utilizers.

The initial oxidative attack on propane can occur either via terminal or subterminal oxygen insertion. Although the metabolism of propane has been studied in more detail compared with C_2-C_4 gaseous hydrocarbons, it is still not clear whether propane is oxidized by a mono or di-oxygenase system. Terminal and subterminal oxidation pathways were reported in several microorganism. If the terminal carbon atom is oxidized, propan-1-ol will be produced which would be further oxidized to propanal and then to propionate (Fig. 1.4). The latter could then be metabolized to methylmalonate and then to succinate (Fig. 1.5) (Vestal and Perry, 1969). Subterminal oxidation could occur by two possible pathways, both of which results in oxidition of propane to propan-2-ol and then to acetone. Subsequently, the latter could be oxidized to methyl acetate and then to acetate and methanol (Fig. 1.6.) or to acetol (Fig. 1.7). The latter could be metabolized either to pyruvate via pyruvaldehyde (see Taylor *et al.*, 1980) or to acetate and formaldehyde (Fig. 1.8) via

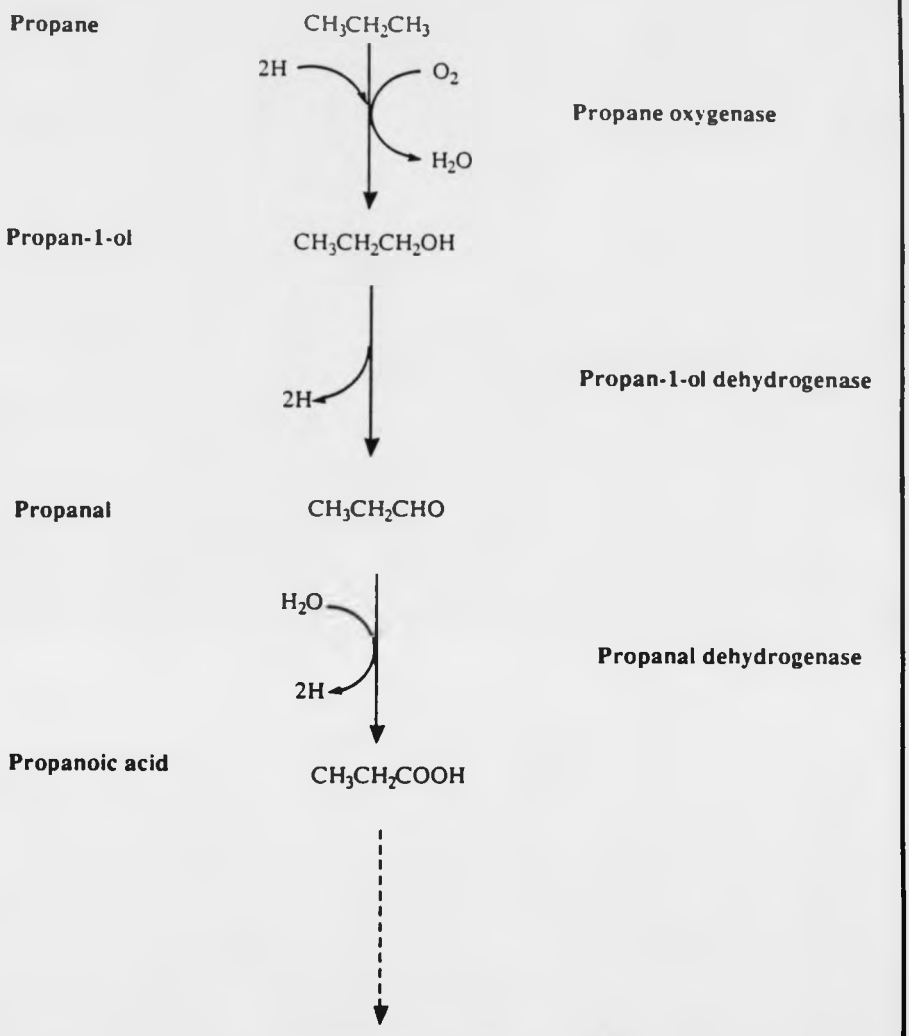

Figure 1.4 Terminal oxidation of propane

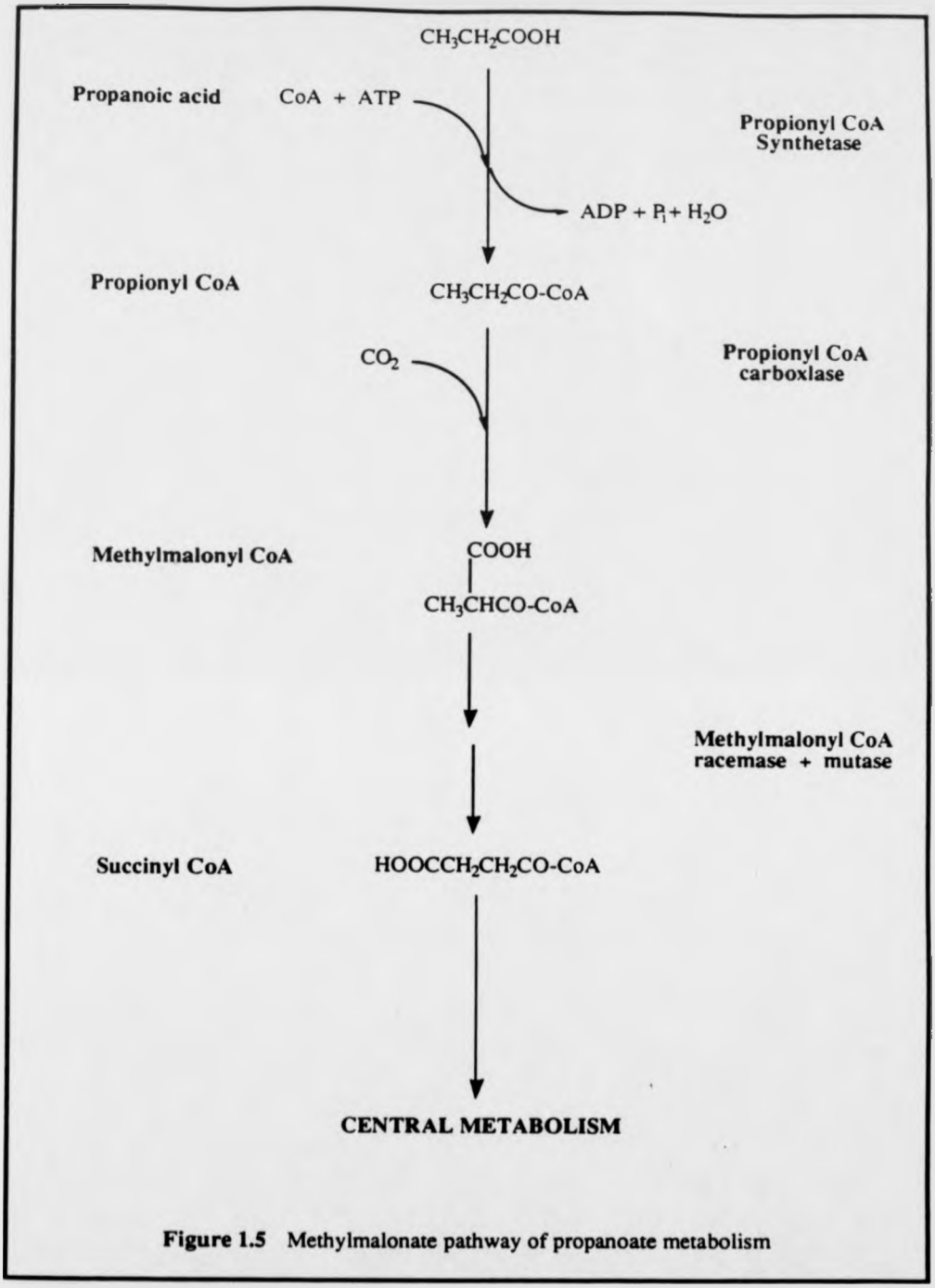

Figure 1.5 Methylmalonate pathway of propanoate metabolism

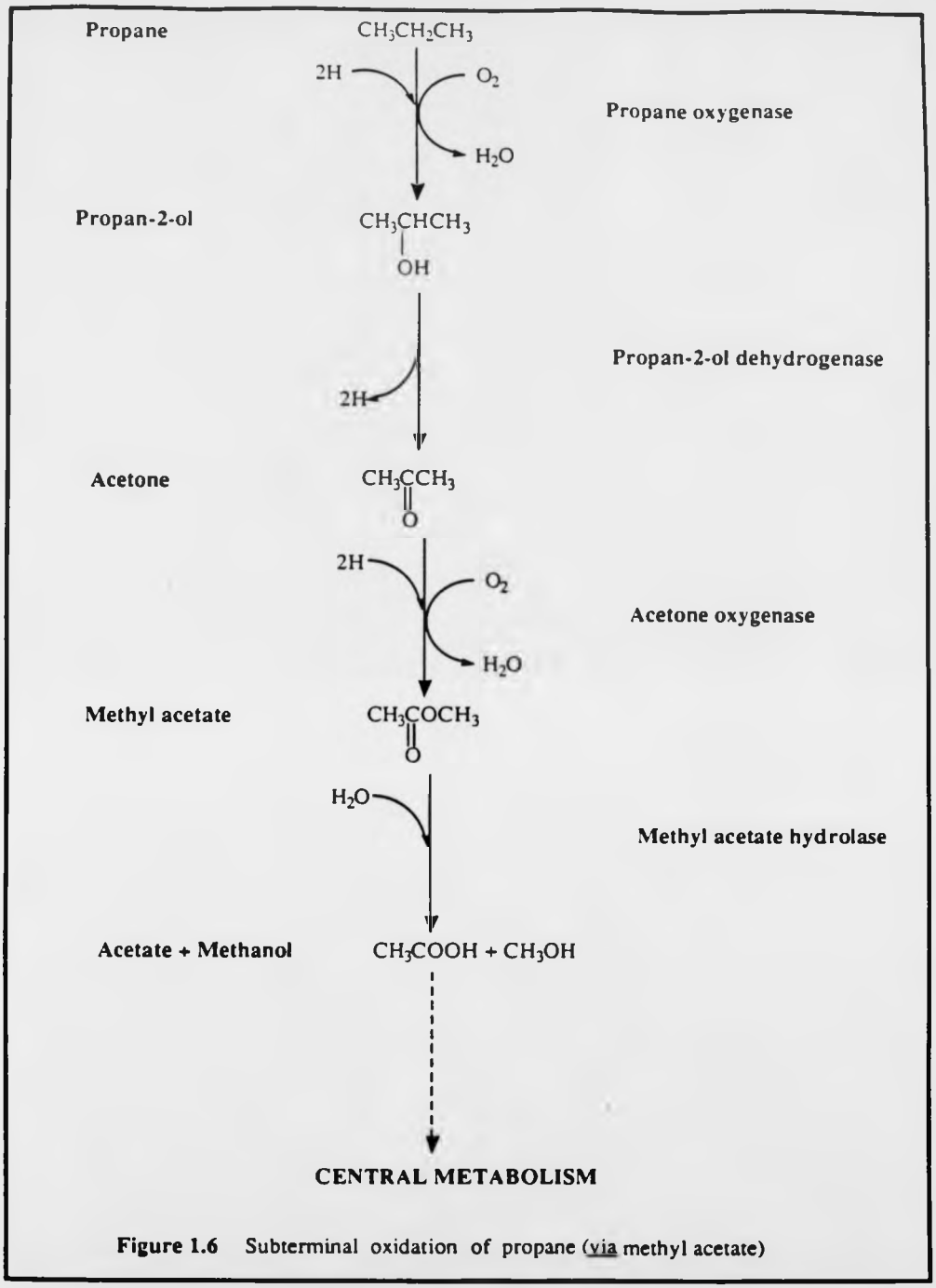

Figure 1.6 Subterminal oxidation of propane (via methyl acetate)

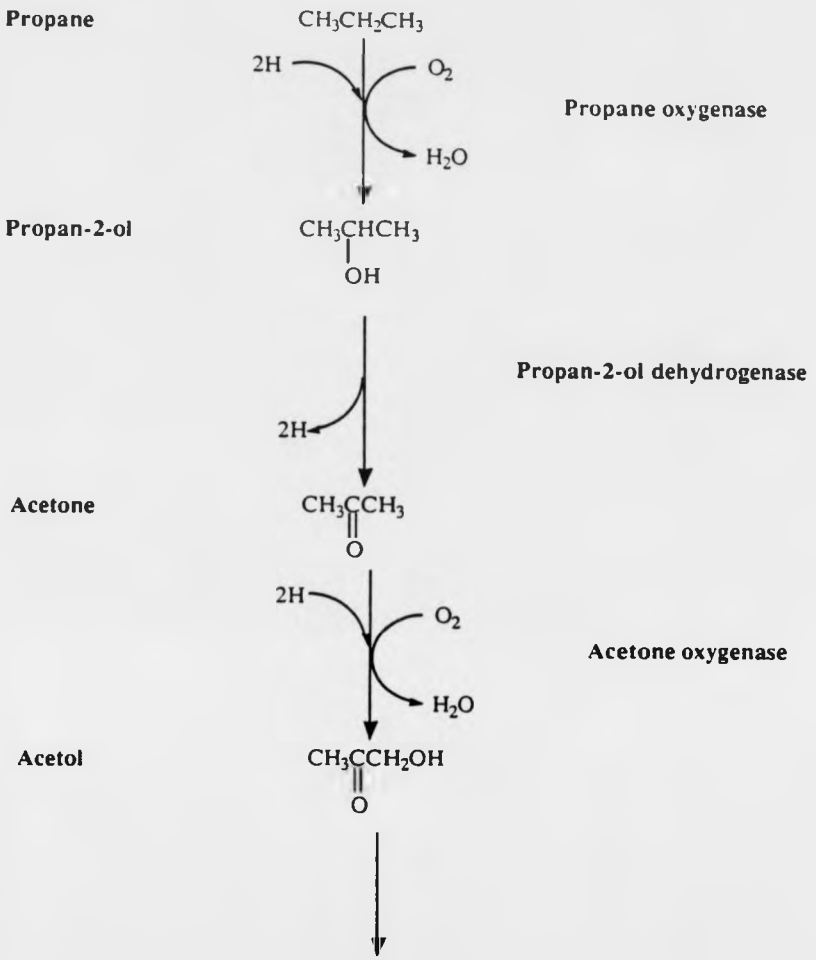

Figure 1.7 Subterminal oxidation of propane (via acetol)

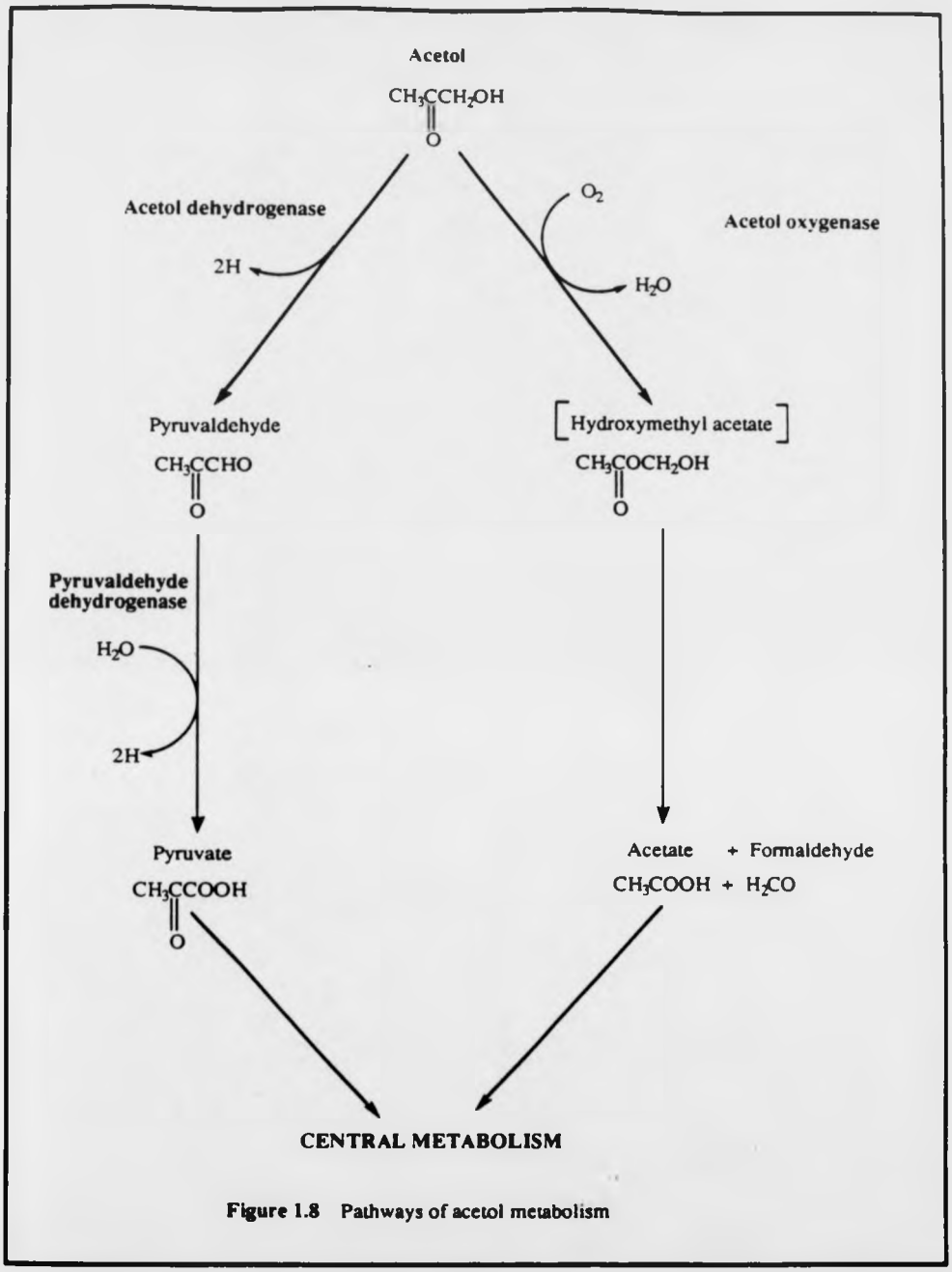

Figure 1.8 Pathways of acetol metabolism

the unstable intermediate hydroxymethylacetate (see Hartmans and deBont, 1986).

The work of J.J. Perry and his colleagues with propane-utilizing mycobacteria, M.vaccae JOB5, M.smegmatis and M.convolutum R-22 has resulted in a greater understanding in the metabolism of propane. They concluded that the major route of propane assimilation is via the subterminal oxidation of propane. This conclusion has been challenged by Stephens (1983) and Woods (1988).

1.3.3.2 The work of Perry *et al.*

Perry (1968) studied the substrate specificity of three bacteria that are capable of growth on propane and on a wide variety of hydrocarbons. One of these isolates, M.vaccae JOB5, was studied in most detail. Respirometric studies were performed using a Warburg apparatus to measure oxygen uptake by a resting cell suspension. Propan-2-ol and acetone-grown JOB5 oxidized propane readily without an induction period. This phenomenon supports the idea of implicating these two compounds as intermediates in propane metabolism. The suggestion was however criticized by Stephens (1983) and Woods (1988). Their main objection was based on the observation that propane-grown cells oxidized acetone at low rate compared with other intermediates of the terminal and subterminal oxidation pathway.

Vestal and Perry (1969), investigating the metabolism of propane using the same organism, performed ^{14}C radiotracer experiments. They suggested that propane is not metabolized *via* terminal oxidation due to the presence of isocitrate lyase activity in propan-2-ol, acetate, or propane grown cells and the absence of this inducible enzyme in propan-1-ol and propanoate grown cells. Thus, they stated that propane could be metabolized *via* acetate (Fig. 1.9). However, sodium arsenite was used in further experiments to cause an accumulation of labelled pyruvate that was recovered from $^{14}CO_2$. Propanoate-grown cells accumulated a significant amount of pyruvate, whereas propane and propan-2-ol accumulated relatively little pyruvate. These results led them to suggest that a CO_2 fixation reaction is involved in propanoate metabolism and propane was being metabolised *via* acetate. This along with the isocitrate lyase results were criticized by Stephens (1983) for the following reasons. Firstly, the presence of isocitrate lyase in propane and propan-2-ol-grown cells does not preclude oxidation *via* pyruvate. Secondly, the presence of isocitrate lyase in propane and propan-2-ol grown cells does not interfere with the possibility of implicating propanoate as an intermediate in propane metabolism since this enzyme is required in the pathway of propanoate. Thirdly, in the presence of arsenite, a significant amount of 2-^{14}C-propan-2-ol was apparently being converted to pyruvate. This suggests that arsenite inhibited one or more of the reactions involved in the initial steps of propan-2-ol oxidation.

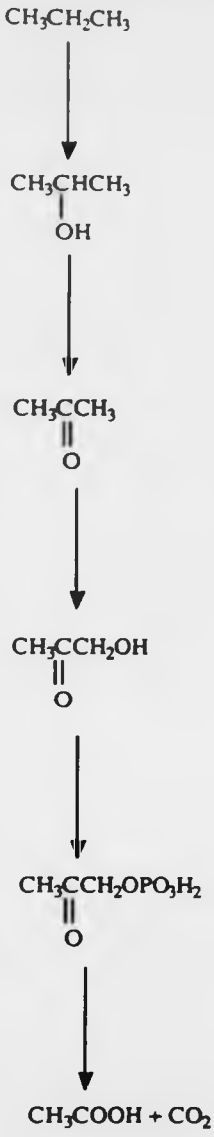

Figure 1.9 Possible pathways of propane metabolism by M. vaccae JOB5 (adapted from Perry, 1980).

N.B Sources of oxidizing and reducing equivalents not given

Vestal and Perry (1971) studied the presence of odd and even chain length fatty acids from propane, propan-2-ol, propanoate and acetate-grown M.vaccae JOB5. They detected a considerable amount of odd chain length fatty acids in propane grown cells, indicating terminal oxidation of propane via propanoate.

Blevins and Perry (1972) studied the metabolism of propane, n-propylamine and propanoate by Mycobacterium convolutum R-22. They used similar methods to those used with M.vaccae JOB5. Results obtained from respiration rates, enzyme assays and $^{14}CO_2$ incorporation experiments suggested to them that propane is metabolised via acetone, and propanoate and n-propylamine are metabolised via the methylmalonate pathway. In the presence of sodium arsenite, $^{14}CO_2$ was incorporated into pyruvate in propanoate- and n-propylamine-grown cells, whereas a negligible amount of labelled pyruvate was produced when cells grown on propane or acetone. Propane-grown R-22 oxidized acetone at a greater than propan-1-ol and propan-2-ol. In contrast, propanoate was oxidized very slowly. This phenomenon does not rule out oxidation of propane via propanoate due to a lack of induction of uptake system.

Coleman and Perry (1984) studied the fate of the C_1 moiety that was cleaved from acetol (into a C_2 and a C_1 fragment) by propane-utilizing M.vaccae JOB5. It was suggested that this was released as CO_2 (Vestal and Perry, 1969) (Fig. 1.9). Coleman and Perry measured the evolution of $^{14}CO_2$ from

either 2-^{14}C-acetone or 1,3-^{14}C-acetone by propane-grown JOB5. They found that both substrates released the same rate of $^{14}CO_2$. M.vaccae JOB5 was grown on propan-2-ol in the presence of 1-^{14}-acetate, ^{14}C-bicarbonate, ^{14}C-formaldehyde or 1-^{14}C-1,2-propanediol. The 1,2-propandiol (1,2-PDL) was used instead of acetol as it is supposedly metabolized via acetol. The percentage of the total incorporation of labelled ^{14}C into lipid, nucleic acid and protein was determined. Results showed that the incorporation level of labelled 1,2-PDL was very close to that obtained from formaldehyde. In addition, a higher percentage of labelled guanine was observed in cells that were exposed to labelled 1,2-PDL and formaldehyde than did those which were exposed to radiolabelled acetate or biocarbonate. Also, 40% of the guanine's carbon was derived from reduced C_1. These results indicate that acetol is cleaved into acetate and a reduced C_1 moiety (formaldehyde) through an unstable intermediate, hydroxymethylacetate. Hartmans and de Bont (1986) proposed the same pathway for acetol metabolism by Mycobacterium Py1 (see section 1.3.3.4).

1.3.3.3 Other work on propane metabolism

1.3.3.3.1 The metabolism of propane by various bacterial species

Lukins and Foster (1963) investigated the metabolism of propane by Mycobacterium smegmatis 422. Cells were grown on n-alkane then washed and resuspended in mineral salts medium

lacking a nitrogen source and incubated with n-alkane for 15 hours. Only methylketones were detected in the supernatant. This indicated that only a subterminal oxidation pathway was involved in the metabolism of propane. However, this type of experiment does not rule out the possibility of terminal oxidation in propane metabolism. Simultaneous adaptation experiments showed that propane and acetone were only oxidized by propane and acetone-grown cells whereas, propan-1-ol was not oxidized by these cells. This suggests that acetone-grown cells were "back-adapted" to propane.

Smirnova and Taptykova (1967) studied the ability of 22 strains of the genus Nocardia to utilize methane, ethane and propane. Nine cultures (41%), belonging to seven species were able to grow on propane. Only one strain showed weak growth on methane. Except for one strain which did not grow on propanal, all species were able to grow on both propanal and propanoate. They also were capable of growth on propane after they had been grown on propanal, propanoate or acetone. Still, the metabolism of propane cannot be ascertained from these types of experiments.

Pabst and Brown (1967) isolated a Mycobacterium sp. that utilized ethane and propane, but not n-butane or isobutane. It also had the ability to grow on propan-1-ol and propan-2-ol. Simultaneous adaptation studies showed that propane was oxidized by propane- and propan-2-ol-grown cells, but not propan-1-ol-grown cells. However, propane-grown cells oxidized propane, propan-1-ol and acetone, although the

organism could not grow on the later. Cell suspensions of propane-grown Mycobacterium were supplied with propane in addition to acetone or propan-1-ol. After they analysed culture supernatants, Pabst and Brown found that propan-2-ol was the only product detected. They suggested that propane was oxidized to propan-2-ol and then to acetone , but no clear evidence for this pathway was provided.

Babu and Brown (1984) studied the metabolism of propane and isobutane by Nocardia paraffinicum (Rhodococcus rhodochrous). Propane-grown cells produced a considerable amount of propan-1-ol whereas, the amounts of propan-2-ol and acetone produced were negligible. Further studies using $^{18}O_2$ and $H_2{}^{18}O$ showed that only $^{18}O_2$ was incorporated into propan-1-ol, indicating that an oxygenase system was present. They also observed that a 2:1 ratio of hydrocarbon to oxygen was consumed by propane or isobutane-grown cells. This indicates the involvement of a dioxygenase which catalyzed the oxidation of two molecules of propane with one oxygen molecule to yield two molecules of C3 alcohol. Unfortunately, these results suggest the involvement of an a monooxygenase system in R.rhodochrous.

Stephens and Dalton (1986) isolated 30 strains of propane-utilizing bacteria. They were classified into three groups according to their ability to grow on acetone. Three strains (B3aP, PrIO3 and B2) which belong to the genus Arthrobacter were selected for detailed study. Strain PrIO3 grew slowly on acetone whereas strain B3aP could not. Propane-grown B3aP

and PrIO3 could oxidized propan-1-ol, propanal and propan-2-ol but not acetone. Also, both strains excreted acetone during growth on propane. These results suggested that propane was not oxidized *via* the subterminal oxidation pathway. However, strain B2 grew rapidly on acetone. Propane-grown B2 oxidized propan-1-ol, propanal, propan-2-ol and acetone. Similarly, acetol was rapidly oxidized by acetone and propan-2-ol-grown cells. This indicated that propane was oxidized in this strain *via* terminal and subterminal oxidation pathways. Nevertheless, experiments using cell-free extract assays to detect the accumulation of alcohols by propane monoxgenase were unsuccessful because of the organism's resistance to breakage by both physical and chemical methods. All three strains were able to oxidize methanol and formaldehyde after growth on propane. This could be due to "the action of non-specific alcohol and aldehyde dehydrogenase involved in propane metabolism" (Stephens and Dalton, 1986).

MacMichael and Brown (1987) studied the role of CO_2 in the metabolism of propane by *Rhodococcus rhodochrous*. This organism could grow on glucose in the absence of CO_2, but it failed to grow on propane unless CO_2 was provided. This shows that CO_2 was a necessary requirement in propane metabolism. Indeed, Warburg respirometry studies showed that CO_2 was required for the metabolism of terminal intermediates but not for propan-2-ol. Further experiments using 2-^{14}C-propane and $^{14}CO_2$ showed that $^{14}CO_2$ was incorporated into labelled pyruvate by propane-grown cells.

In the presence or absence of sodium arsenite, a significant amount of labelled pyruvate was accumulated when propane-grown cells were exposed to $^{14}CO_2$ along with propan-1-ol, propanal and propanoate. These results led them to the suggestion that propane was being metabolised *via* terminal oxidation involving the methylmalonate pathway (see Fig. 1.4 & Fig. 1.5).

1.3.3.3.2 *The metabolism of propane by R.rhodochrous PNKb1*

Recently, more information on the physiology, biochemistry and genetics of propane-utilizing *Rhodococcus rhodochrous* PNKb1 has been accumulated from the work of Woods (1988) and Ashraf (1990).

Woods and Murrell (1989) isolated over 80 strains of Gram-positive propane-utilizing bacteria that appeared to be members of the CMN complex. Owing to its rapid growth on propane as the sole source of carbon and energy, *Rhodococcus rhodochrous* PNKb1 was selected for detailed studies. Furthermore, it grows on most of the potential intermediates in propane metabolism except methyl acetate, methanol and methylglyoxal (pyruvaldehyde). Simultaneous adaptation experiments demonstrated that PNKb1 could oxidize both terminal and subterminal intermediates.

A novel propane-specific oxygenase has been described. SDS-PAGE of cell-free extracts of cells grown on propane and various potential intermediates in propane metabolism

pointed to the presence of three major polypeptide bands of 69, 59 & 57 kDa found only in propane-grown cells. Thus, Woods and Murrell concluded that these polypeptides were "likely to be components of the propane oxygenase system". However, similar polypeptides were observed in SDS-PAGE of cell-free extracts of acetol-grown cells (Ashraf, 1990). He measured the specific activities of "key enzymes" of propane oxidation in cell-free extracts of propane and pyruvate-grown cells. NAD^+-linked propan-1-ol dehydrogenase activity in propane-grown cells showed little increase compared with that in pyruvate grown cells. However, other enzymes of the terminal oxidation pathway showed four- to five- fold increase in the level of activity in propane-grown cells compared to those obtained with pyruvate. Likewise, NAD^+-dependent propan-2-ol dehydrogenase activity showed a four-fold increase in propane-grown cells whereas acetol oxygenase activity was absent from pyruvate-grown cells and present in propane-grown cells. The absence of acetol dehydrogenase activity and the presence of acetol oxygenase may indicate that acetol is not metabolised *via* pyruvate.

The propane-specific oxygenase activity was measured by the ability of PNKb1 to epoxidate propene (propylene) to 1,2-epoxypropane (propylene oxide). The propylene oxide was not further metabolised by PNKb1 and could be detected by gas chromatography (Woods and Murrell, 1989). However, addition of propane to the assay system inhibited the epoxidation. This led them to suggest that "propane and propene were

competing for the same active site". Nevertheless, this conclusion cannot be definitely made using this type of assay. Attempts to purify propane oxygenase were unsuccessful due to its instability in the cell-free extracts ($T_{1/2}$ = 150 minutes). However, the effect of potential inhibitors on the propane-specific oxygenase activity in cell-free extracts and resting cell suspension of propane-grown PNKb1 was investigated. The identical results obtained with both propane and propene indicated that epoxide formation was indeed catalysed by the propane oxygenase system. Carbon monoxide does not show any inhibition whatsoever, demonstrating the lack of involvement of P_{450}-type cytochromes.

Ashraf and Murrell (1990) detected NAD^+-dependent primary and secondary alcohol dehydrogenase in the soluble fraction of cell-free extracts of propane-grown R.rhodochrous PNKb1. Propane and propan-2-ol-grown cells, but not propan-1-ol, showed elevated levels of primary and secondary alcohol dehydrogenase activity. This indicates that the metabolism of propane may proceed via terminal and/or subterminal oxidation. Secondary alcohol dehydrogenase was purified using a two-step procedure. This enzyme exhibited both primary and secondary NAD^+-linked alcohol dehydrogenase activity. The Mr of the enzyme was approximately 86,000 and was composed of two identical subunits of Mr 42,000. This enzyme was similar in size and subunit composition to other NAD^+-dependent secondary alcohol dehydrogenases from methanol-grown Pichia sp. (Patel *et al.*, 1979) and

Pseudomonas sp. (Hou *et al.*, 1979). However, the properties of this enzyme were found to be different from those described in the current literature. The enzyme showed a broad substrate specificity, oxidizing a range of short chain primary and secondary alcohols (C_2-C_8) and representative cyclic and aromatic alcohols. Further, the highest activities were observed with secondary alcohols. *In vivo*, propan-2-ol was converted by alcohol dehydrogenase to acetone. *In vitro*, the latter was converted to propan-2-ol in the reverse reaction at pH 6.5 as follows:

$$\text{Propan-2-ol} + NAD^+ \Leftrightarrow \text{acetone} + NADH^+ + H^+$$

They postulated that secondary alcohol dehydrogenase from PNKb1 could convert various ketones to their corresponding alcohols in the reverse reaction. The enzyme was inhibited by metal complexing agents, indicating that a metal centre may be involved the active site or is necessary for stability of the enzyme. In addition, it was inhibited by iodoacetate, demonstrating the presence of functional thiol groups.

It has been postulated that primary and secondary alcohol dehydrogenase activity was present in extracts of propane-grown cells and cells grown on potential intermediates in propane pathways. It was not clear whether that activity was attributed to one or more enzymes. However, Ashraf and Murrell (1992) isolated several NTG-mutants that were unable to utilize propan-1-ol (*alcA*$^-$), propan-2-ol (*alcB*$^-$) or either propan-1-ol & propan-2-ol (*alcAB*$^-$) as the sole source of carbon and energy. All three classes of mutants failed to

grow on propane as their sole source of carbon and energy. The isolation of *alc*A$^-$ and *alc*B$^-$ phenotypes indicated that two alcohol dehydrogenases are required for the metabolism of propane. The inability of *alc*A$^-$ or *alc*B$^-$ mutants to grow even slowly on propane suggests that a mixture of propan-1-ol and propan-2-ol is produced in the metabolism of propane.

Antibodies against NAD^+-dependent secondary alcohol dehydrogenase were raised and used in Western-blotting of cell-free extracts of cells grown on propane and potential intermediates in propane metabolism. This enzyme was only synthesized after growth on propane, propan-2-ol and acetol. This also testifies that another alcohol dehydrogenase may be present in the pathways of propane. This enzyme was also synthesized by other propane-utilizing *Rhodococcus*-*Nocardia* complex bacteria after growth on propane (Ashraf, 1990). The above results provide firm evidence that terminal and subterminal oxidation pathways are implicated in the metabolism of propane by *R.rhodochrous* PNKb1.

In relation to propane metabolism, it is worth devoting the next sections to a discussion of the metabolism of 1,2-propanediol, acetone and acetol.

1.3.3.4 The metabolism of 1,2-propanediol

1,2-Propanediol (1,2-PDL) was not reported in the literature to be an intermediate in the propane oxidation pathway. It was however suggested that it is metabolized *via* acetol

(Levine and Krampitz, 1952 and Hartmans and de Bont, 1986). At the same time there are other possibilities for the metabolism of 1,2-PDL under aerobic conditions.

Willetts (1979) described a Flavobacterium sp. NCIB 11171 that metabolized 1,2-PDL aerobically to lactaldehyde and then to pyruvate. However, some 1,2-PDL was catabolized under microaerophilic conditions to propanal and then reduced to propan-1-ol. In Nocardia sp., 1,2-PDL was metabolized to propanal, propionate and then to succinate (de Bont *et al.*, 1982). Elevated levels of diol dehydratase were detected during growth on propylene oxide and 1,2-PDL. Jetter (1990) described Salmonella typhimurium that grew aerobically on 1,2-PDL as the only source of carbon and energy. This bacterium did not grow on 1,2-PDL unless cobalamin was provided which suggested that the metabolism of 1,2-PDL is dependent upon the provision of cobalamin as a nutritional supplement. Jetter isolated thirty-three insertion mutants which could not grow on 1,2-PDL, but could still grow on propionate. However, diol dehydratase activity was absent in some of the mutants.

A novel Pseudomonas sp. strain TB-135 was capable of utilizing 1,2-PDL under aerobic conditions to lactaldehyde (or acetol), D-lactic acid, and then to pyruvaldehyde (Shigeno and Nakahara, 1991).

1.3.3.5 The metabolism of acetone

Lukins and Foster (1963), Vestal and Perry (1969) and Coleman and Perry (1984) suggested subterminal oxidation in the metabolism of propane, with acetone as an intermediate. Stephens and Dalton (1986) detected a considerable amount of acetone in the supernatant of propane-grown Arthrobacter sp. The metabolism of acetone has been studied by several groups of investigators who proposed that acetone under aerobic conditions is cleaved into organic C_1 and C_2 fragments (Levine and Krampitz, 1952; Lukins and Foster, 1963; Vestal and Perry, 1969 and Taylor *et al.*, 1980).

Levine and Krampitz (1952) showed that acetol was an intermediate in the oxidation of acetone and it was further metabolized to acetaldehyde and a C_1 moiety. They also indicated that acetone and 1,2-PDL are metabolized via acetol.

Lukins and Foster (1963) reported that methylketones were intermediates in the metabolism of propane by Mycobacterium smegmatis 422. They also demonstrated that acetone can be oxidized to acetol. They presented no clear evidence to indicate how acetol is further metabolized.

Vestal and Perry (1969) studied the metabolism of propane by Mycobacterium vaccae JOB5. They exposed non-proliferating propane-grown cells to propane-2-^{14}C. ^{14}C-acetone was the only labeled product obtained. But similar experiments

demonstrated that acetone-2-^{14}C was readily oxidized to acetol.

Another possible route for acetone metabolism was suggested by Taylor *et al.*, (1980). The proposed pathway was as follows: propan-2-ol can be metabolized to acetone and then to acetol (Fig 1.7). The latter could be oxidized to pyruvate via methylglyoxal (Fig 1.8).

Many reports described the anaerobic metabolism of acetone. This can occur by carboxylation of acetone to acetoacetate. The latter can then be metabolized by means of direct conversion to acetoacetyl-CoA, followed by reduction to ß-hydroxybutyrate (Platen and Schink, 1987; Bonnet-Smits *et al.*, 1988; Platen and Schink, 1989).

1.3.3.6 The metabolism of acetol

In 1941, Goepfert pointed to the formation of formaldehyde and acetol (1-hydroxyacetone) from propan-2-ol, 1,2-PDL and acetone-grown Fusarium sp. However, many investigators reported that acetol was an intermediate in the metabolism of propane by certain bacteria (Lukins and Foster, 1963; Vestal and Perry, 1969 and Perry, 1980). Ashraf (1990) showed that acetol is a key intermediate in propane metabolism by Rhodococcus rhodochrous PNKb1. There are two proposed pathways for the metabolism of acetol (Fig. 1.8). Taylor *et al.* (1980) suggested that acetol could be oxidized by acetol dehydrogenase to methylgloxal and then to

pyruvate. However, Hartmans and de Bont (1986) studied the metabolism of acetol by Mycobacterium Py1. They proposed that acetol could be oxidized by acetol monooxygenase to acetate and formaldehyde through an unstable intermediate hydroxymethylacetate (Fig. 1.8). The activity of acetol monooxygenase was detected in cell-free extracts as well as in the supernatant of cells grown on 1,2-PDL and acetol (Hartmans and de Bont, 1986).

1.3.4 The metabolism of butane

McLee *et al.* (1972) isolated fifteen bacterial strains mainly belonging to Arthrobacter sp. and Brevibacterium and four moulds which were capable of growth on butane. They investigated the optimal growth conditions on butane. The effect of varing gaseous atmosphere on growth rates studies, indicated that no increase in the growth rate was observed when the air:butane ratio was increased. However, high oxygen partial pressures inhibited most of the bacterial growth. Two Arthrobacter strains (AK 19 & J) were selected for further studies by Kormendy and Wayman (1974). Butane and butan-1-ol-grown cells showed the presence of unusual intracellular structures which were absent when cells were grown on glucose. These structures appeared as a round body, which they called an "oxisome", surrounded by a sphere of electron-transparent material. The latter was connected to the cytoplasm by a fairly wide bridge and by several finer cytoplasmic strands.

The metabolism of butane was initially studied by M.vaccae JOB5 (Vestal and Perry, 1968).They proposed that butane was metabolised via acetate. Further studies using the same organism were carried out by Phillips and Perry (1974) investigating the metabolism of butane and butane-2-one. They suggested that butane was terminally oxidized due to the induction of isocitrate lyase activity after growth on n-butane, butyrate, ß-hydroxybutyrate and acetate (Fig. 1.10). Nevertheless, butan-2-one was cleaved into C_3 and C_1 and metabolised via propanoate involving the methylmalonate pathway. Radiotracer studies showed that butan-2-one and propanoate-grown cells accumulated labelled pyruvate from the incorporation of $^{14}CO_2$. Moreover, butane, butanoate and acetate-grown cells did not accumulate pyruvate, implying that butan-2-one was not an intermediate in the butane oxidation pathway.

Stephens (1983) studied the metabolism of butane by Arthrobacter strain B3aP. This strain grows on all potential terminal intermediates in the butane oxidation pathway but not on subterminal intermediates, such as butan-2-ol and butanone. Simultaneous adaptation experiments showed that butane-grown cells oxidized both potential intermediates of the terminal and subterminal oxidation pathways, although the rate of butanone oxidation was fairly low. Also, butanone was excreted from butane-grown cells. These results indicated that a small proportion of butane may be oxidized via subterminal oxidation to butanone, which is not further metabolised in this strain.

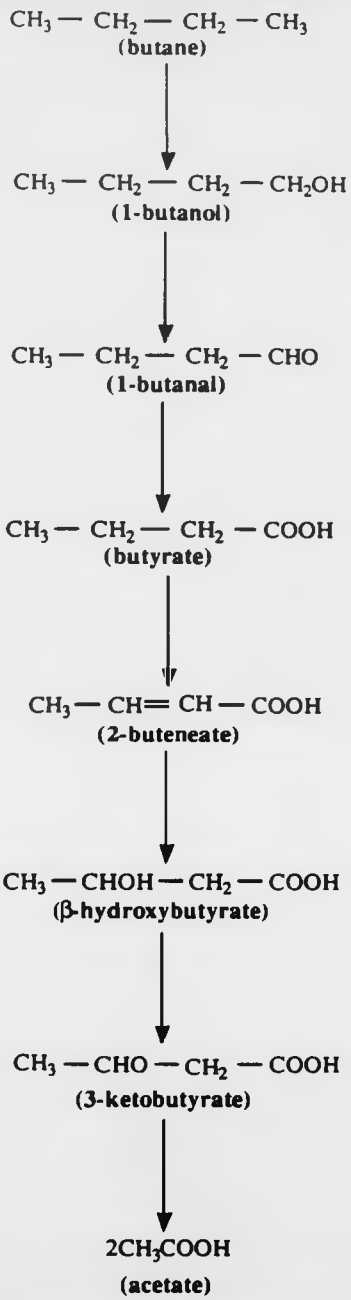

Figure 1.10 Terminal oxidation of butane (adapted from Atlas, 1984)

N.B Sources of oxidizing and reducing equivalents not given

Van Ginkel *et al.* (1987) measured the activity of isocitrate lyase by butane-grown Nocardia TB1. In the presence of sodium arsenite, butanoate was excreted by a butane-grown resting cell suspension. This led them to suggest that butane was oxidized via the terminal route.

1.4 Enzymology of n-alkane metabolism

1.4.1 Oxygenases

Enzymes that incorporate an oxygen molecule into a substrate are known as "oxygenases". These enzymes have been divided into mono- and di-oxygenases depending on whether both or just one atom of oxygen molecule is incorporated into the substrate (Hayaishi *et al.*, 1975).
Monooxygenases are the most common among oxygenases in the metabolism of methane, higher gaseous alkane and liquid n-alkanes. Methane monooxygenase (MMO) from M.capsulatus (Bath) has been purified (Colby and Dalton, 1978). A brief background of the biology of MMO was discussed in section 1.2.2 (For more detail see Dalton and Leak, 1985; Dalton, 1992 and Dalton *et al.*, 1993).

1.4.1.1 Propane "oxygenase" activity

Very little is known about the enzymology of propane metabolism. In fact, there are no reports whatsoever that describe the purification of a propane oxygenase system. However, Perry (1979) suggested that propane oxygenase is an

inducible enzyme. Propane-grown M.vaccae JOB5 does not support growth on cyclohexane, although propane-grown cells cooxidized cyclohexane to cyclohexanone. This led them to the assumption that M.vaccae JOB5 possessed a propane monooxygenase system "PMO" for the metabolism of propane.

Patel *et al.* (1983) demonstrated that cell-free extracts (particulate fraction) from Arthrobacter sp. CRL-60 and soluble extract derived from P.fluorescens NRRL B-1244 and Brevibacterium sp. NRRL B-11319 catalyze the oxygen and NADH-dependent hydroxylation of ethane, propane and butane. Both primary and secondary alcohols built-up in the extracts, suggesting to the involvement of a monooxygenase-type system in these alkane metabolism pathways.

A novel "propane intermolecular dioxygenase" was described by Babu and Brown (1984) in Rhodococcus rhodochrous. Yet, no attempts were made to measure the activity in cell-free extracts. As previously mentioned, a propane specific oxygenase system was described in R.rhodochrous PNKb1 (see section 1.3.3.3.2). However, no firm evidence was given to indicate that this was a propane mono- or di-oxygenase.

1.4.1.2 Octane monooxygenase

The enzyme system of octane monooxygenase from P.oleovorans (putida) is very similar to that from P.aeroginosa (Van Eyk and Bartels, 1970 and Matsuyama *et al.*, 1981). This system consists of three components; NADH-rubredoxin reductase,

rubredoxin and ω-hydroxylase (Peterson *et al.*, 1966). The conversion of octane to octan-1-ol needs the transfer of 2 electrons from NADH to oxygen (further evidence was demonstrated by Peterson *et al.*, 1967). Then, the 2 electrons are transferred to the non-haem iron prosthetic group of rubredoxin (Peterson *et al.*, 1966 and Ueda *et al.*, 1972). The reductase and rubredoxin interact on a 1:1 basis to form a complex and the redox reaction occurs (Peterson *et al.*, 1967) (Fig. 1.11). This system is common among various Pseudomonas species. However, a different system was described in a Corynebacterium sp. 7EIC. Soluble cell-free extracts of octane-grown cells could oxidize octane to a mixture of octan-1-ol and octanoate in an oxygen and NADH-dependent reaction. This system was inhibited by carbon monoxide, indicating the involvement of cytochrome P_{450} in this organism. For a recent review on cytochrome P-450 dependent monooxygenase, see Mansuy *et al.* (1989).

1.4.1.3 Ketone monooxygenase

Britton and Markovetz (1977) purified a ketone monooxygenase from tridecan-2-one grown Ps.cepacia. Undecyl acetate was formed from tridecan-2-one in an oxygen and NADH-dependent reaction.

An NAD(P)H-dependent cycloketone oxygenase activity was also detected in Nocardia sp. after growth on cyclohexanone (Hasegawa *et al.*, 1983).

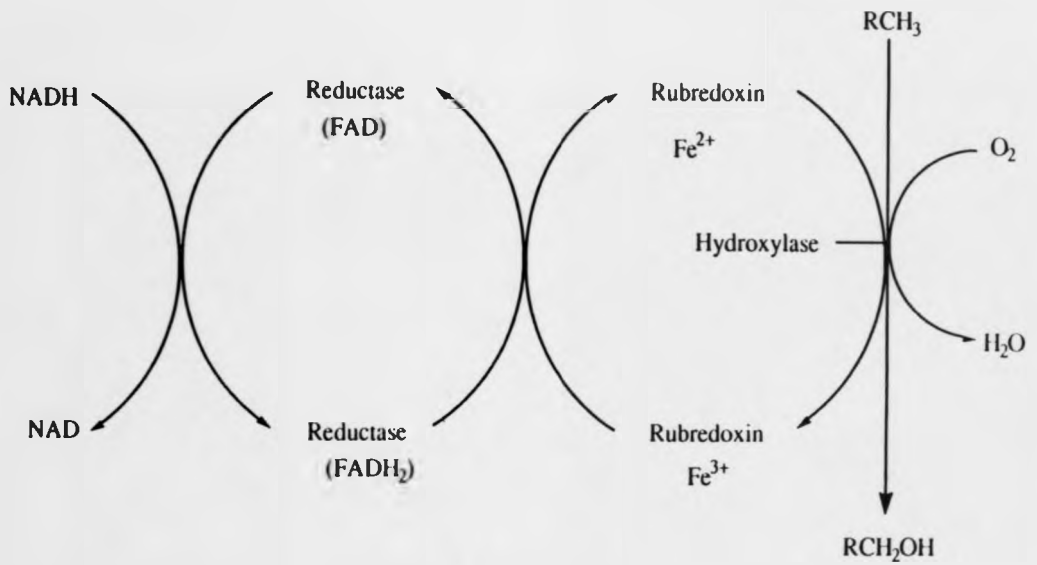

Figure 1.11 Postulated pathway of electron transfer in octane monooxygenase from P.oleovorans (from Ratledge, 1978)

Several groups of investigators suggested that acetone was hydroxylated to acetol (see section 1.3.3.5). However, acetone monooxygenase was not detected in cell-free extracts of acetone-grown Corynebacterium sp. (Taylor *et al.*, 1980) They raised two logical reasons for the interpretation of this phenomenon: it could be due to the extreme lability of the enzyme or a requirement for structural organization that was lost upon cell rupture. Also, Woods and Murrell (1989) were unable to detect acetone monooxygenase activity in cell-free extracts of propane-grown R.rhodochrous PNKb1.

1.4.1.4 Acetol monooxygenase

A novel soluble NADPH-dependent acetol monooxygenase was detected in acetol and 1,2-propanediol-grown Mycobacterium Py1 but not acetone-grown cells (Hartmans and de Bont, 1986). However, acetate and formaldehyde accumulated in cell-free extracts of acetol-grown cells, implying that acetol is oxidized by acetol monooxygenase to acetate and formaldehyde via unstable intermediate, hydroxymethyl acetate (Fig. 1.8). Acetol monooxygenase was totally inhibited by carbon monoxide and Cu^{2+} but not cyanide, indicating that a cytochrome P_{450}- type oxygenase might be involved.

1.4.2 Alcohol dehydrogenase(s)

1.4.2.1 Alcohol dehydrogenase from propane utilizers

Secondary alcohol dehydrogenase from R.rhodochrous PNKb1 has been purified and fully characterized (Ashraf, 1990). For a detailed description see section 1.3.3.3.2.

NAD^+-linked primary and secondary alcohol dehydrogenase activity was detected in cell-free extracts of propane-grown Pseudomonas fluorescens (Hou *et al.*, 1983). Secondary alcohol dehydrogenase was purified to homogeneity. The Mr of the enzyme was approximately 144.5 kDa and consisted of four identical subunits. The K_m value for propan-2-ol was 8.5 X 10^{-5} M. The enzyme was totally inhibited by metal-complexing agents and thiol reagents. A similar enzyme in terms of size and subunits structure was purified by Coleman and Perry (1985) from Mycobacterium vaccae JOB5 after growth on propane using a three step procedure. It had a molecular weight of 136 KDa with identical subunits of Mr of 37,000. The iso-electric point was 4.9. NAD, but not NADP, could act as electron acceptor. The K_m value for propan-2-ol and NAD were 4.9 X $10^{-5}$and 2.8 X 10^{-4} M respectively. This enzyme was also inhibited by thiol reagents and metal chelators.

1.4.2.2 Alcohol dehydrogenase from butane-utilizers

Beers (1988) purified four alcohol dehydrogenases from Ps.butanovora. Three enzymes were soluble and NAD^+-dependent, one being a primary alcohol dehydrogenase and the

two others being secondary alcohol dehydrogenases. The fourth enzyme was a particulate and $NAD(P)^+$-independent primary alcohol dehydrogenase. The soluble dehydrogenases had a restricted substrate specificity. The particulate enzyme had a wide substrate specificity including methanol and ethanol, but not secondary alcohols.

1.4.3 Aldehyde dehydrogenase(s) from n-alkane-grown bacteria

An $NAD(P)^+$-dependent aldehyde dehydrogenase activity was detected in cell-free extracts of heptane-grown P.aeruginosa. This enzyme was partially purified by Heydeman and Azoulay (1963). Nevertheless, Bertrand *et al.*, 1973 showed both soluble and particulate NAD^+-dependent aldehyde dehydrogenase activity in heptane-grown P.aeruginosa. Both enzymes could oxidize aliphatic C_4 to C_{13} aldehydes.

Guerrillot and Vandecasteele (1977) showed that glucose-grown P.aeruginosa possess two soluble, constitutive, aldehyde dehydrogenases. NAD^+- and $NAD(P)^+$-specific enzymes were partially purified. The former showed high affinity for short- and medium- chain length aldehydes (C_2 to C_{10}) and the $NAD(P)^+$-specific enzyme demonstrated high affinity for longer chain aldehydes (C_9 to C_{14}). However, hexane or heptane-grown cells demonstrate a third particulate, NAD-dependent aldehyde dehydrogenase which had high specific activities for long chain aldehydes. This might implicate a role in alkane metabolism although the role of the constitutive enzymes remained unclear. However, Singer and

Finnerty (1984a) described an $NAD(P)^+$-dependent aldehyde dehydrogenases from Acinetobacter sp. HO1-N that was induced by hexadecane, long chain alcohols and aldehydes but not palmitate or acetate.

1.5 Genetics of n-alkane-utilizing bacteria

A great deal of information about the genetics of hydrocarbon-utilizing bacteria has been accumulated in the past decade. The genetics and molecular biology of obligate methanotrophs has been reviewed by Murrell (1993), and this will not be discussed here. Most of the genetics of n-alkane metabolism has been intensively studied in liquid alkane-utilizing Pseudomonas sp. Thus, a brief background is given in the next section and the reader is also referred to Singer and Finnerty, 1984b.

The genetics of higher gaseous n-alkane oxidation has not been reported in detail in the literature. Recently, unsuccessful studies on the genetics of R.rhodochrous PNKb1 have been reported by Ashraf (1990).

Macham and Heydeman (1974) isolated several NTG-mutants of P.aeruginosa that failed to grow on heptane. Mutant complementation studies demonstrated the presence of three components in the heptane-oxidation system; a membrane-bound component, a labile component and rubredoxin.

The best genetics studies of liquid n-alkane in bacteria were reported in P.putida PpG6 by Neider and Shapiro (1975). This organism was able to grow on C_6-C_{10} alkanes. NTG-generated mutants failed to utilize all five n-alkanes but retained the ability to grow on octanol and nonanol. The genes for the inducible octane hydroxylase system were carried on the OCT plasmid (Chakrabarty *et al.*, 1973). Further studies by Shapiro and co-worker elucidated the structure and regulation of the OCT plasmid. It has been shown to carry alkA, B, C, D, E and R loci (details are summarized in Figs. 1.12 and 1.13). Mutant complementation studies confirmed the presence of an alcohol dehydrogenase locus on the OCT plasmid and indicated the presence of multiple alcohol and aldehyde dehydrogenase loci on the chromosome of P.putida (Grund *et al.*, 1975). A fuller picture of the model of alkane oxidation was proposed by Benson *et al.* (1979) (Fig. 1.13). Also, Owen *et al.* (1984) cloned sequences of the alk operon and characterized them physically and genetically. Further studies were focussed on the cloning of alkBAC, alkR and alkA (For further details the reader is referred to Kok et al. (1989a,b). In contrast, Acinetobacter sp. HO1-N exhibited several significant differences in the alkane oxidation system to those found in P.putida PpG6 (see Singer and Finnerty, 1984b).

1.6 Genetics of R.rhodochrous PNKb1

The genetics of R.rhodochrous PNKb1 was studied by Ashraf (1990). The use of various methods to obtain endogenous

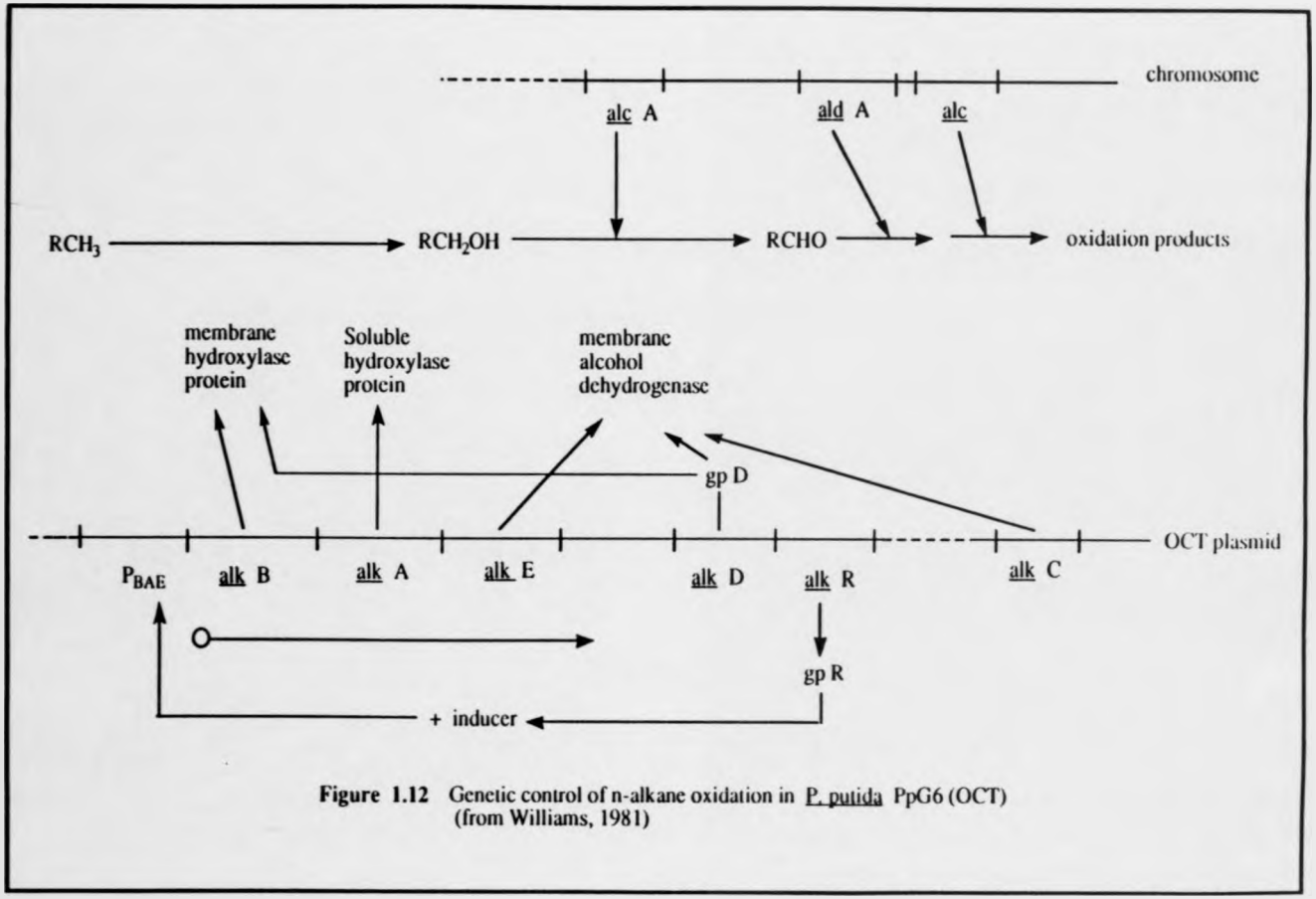

Figure 1.12 Genetic control of n-alkane oxidation in P. putida PpG6 (OCT) (from Williams, 1981)

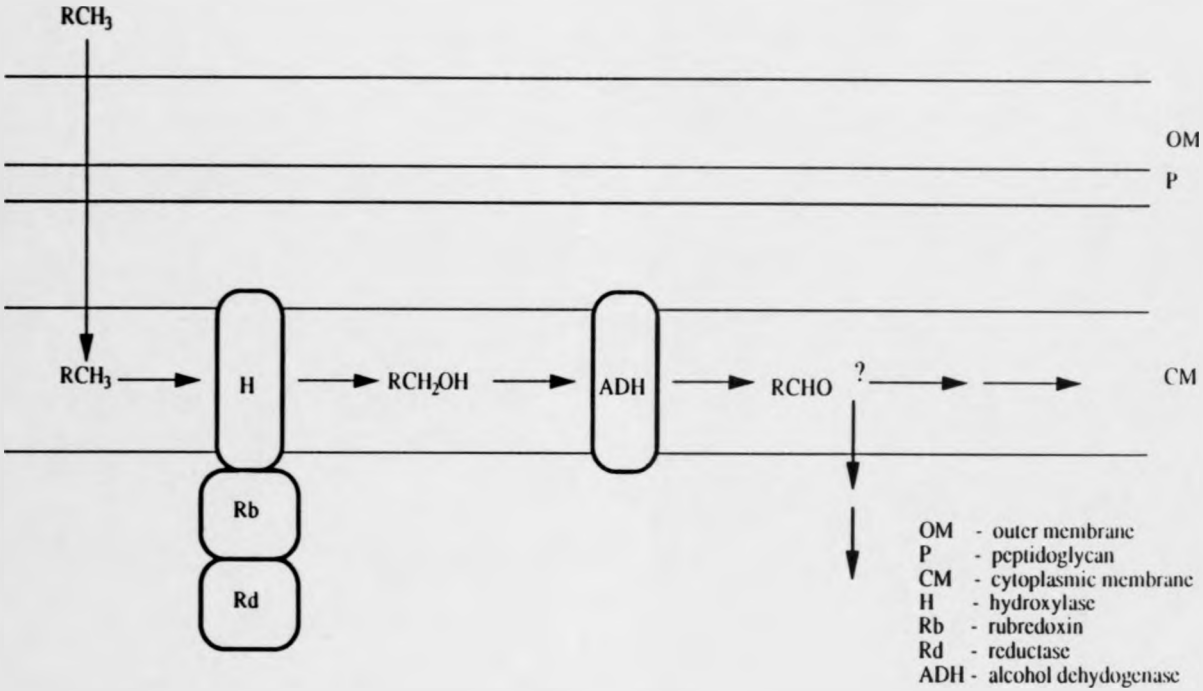

Figure 1.13 Membrane model of n-alkane oxidation in P. putida PpG6 (OCT) (from Benson *et al.*, 1979)

plasmids were unsuccessful. The isolation of plasmid DNA was extremely difficult due to the toughness of the cell-wall. R.rhodochrous PNKb1 showed a broad range of antibiotic resistances. This phenomenon was useful for transformation experiments and for obtaining various mutants. Attempts to develop a transformation system for this organism were unsuccessful. Protoplast transformation was tried using the method of Singer and Finnerty (1988). Microscopic counts examination revealed that 25% of the bacterial population were in the protoplast form, but less than 1% of the protoplasts were regenerated. Control experiments using Rhodococcus sp. H13-A and S.coelicolor gave over 50% regeneration. Similarly, introduction of DNA from Rhodococcus An-1, Str^{r}_{20} to R.rhodochrous PNKb1 failed probably because of the toxicity of aniline to the cells. However, plasmid screening of propane-utilizing bacteria showed that Rhodococcus An-1 does not possess the 90 Kb plasmid. Attempts to transfer a plasmid from E.coli to PNKb1 were also fruitless. Transduction and electroporation techniques used to transfer DNA into PNKb1 gave no significant results (for more details see Ashraf, 1990).

Although the introduction of DNA into PNKb1 using different techniques was unsuccessful, various classes of propane oxidation mutants were obtained by conventional mutagenesis procedures. All mutants failed to utilize propane as growth substrate, but still retained their ability to utilize some of the potential oxidation intermediates in propane metabolism. Propane^{-} (alk^{-}) mutants were unable to

metabolize either propane or acetol, implicating a common enzyme or regulatory element for the metabolism of both substrates. The isolation of propan-1-ol$^-$ (*alc*A$^-$) and propan-2-ol$^-$ (*alc*B$^-$) mutants indicated that both alcohols are intermediates in propane oxidation. The isolation of *alc*AB$^-$ mutants suggested that a mixture of propan-1-ol and propan-2-ol was produced in the metabolism of propane. Acetone$^-$(*Ket*$^-$) mutants failed to grow on acetone, but were still able to utilize propane as growth substrate. This suggests that acetone is probably not an intermediate of propane oxidation.

Two classes of propanoate$^-$ mutants (*Oate*$^-$) were obtained. *Oate*$_1^-$ mutants failed to grow on propane, propan-1-ol, propanal and propanoate, but still retained the ability to utilize subterminal intermediates. This implies that propane and terminal intermediates are metabolised *via* propanoate. *Oate*$_2^-$ mutants failed to utilize propanoate as growth substrate, but succeeded with propane, all other terminal and all subterminal intermediates. This suggests that PNKb1 has a transport system for propanoate uptake.

1.7 *Summary*

The metabolism of liquid n-alkanes is well documented whereas that of ethane, propane and butane are relatively poorly understood. Although the metabolism of propane in *R.rhodochrous* PNKb1 has been investigated in detail, the pathways involved remain unresolved. Isolation and

characterization of *alcA*$^-$, *alcB*$^-$, *alcAB*$^-$ mutants proved that propane is metabolised *via* terminal and subterminal oxidation or a mixture of two pathways. The possibility of propane dioxygenase involvement in PNKb1 was suggested (Ashraf, 1990). Nevertheless, the isolation and characterisation of acetol$^-$ (*aol*$^-$) and 1,2-propanediol$^-$ (*Alc*C$^-$) mutants will indicate whether 1,2-PDL was an intermediate in the metabolism of propane or not. The link between 1,2-PDL and propane pathway(s) needs further attention.

Isolation of a propane oxygenase system was not reported in the literature while acetol monooxygenase was purified (see section 1.4.1.4). Propane oxygenase from *R.rhodochrous* PNKb1 was extremely labile (Woods, 1988). Also, there is no attempt to describe any purification of acetol oxygenase in this organism. SDS-PAGE analysis of cell-free extracts of *R.rhdochrous* PNKb1 grown on propane and potential intermediates of propane oxidation indicated that there might be a common propane/acetol oxygenase system. Two types of ketone monooxygenase were extensively studied. The roles of ketone monooxygenase, alcohol and aldehyde dehydrogenases in relation to alkane metabolism have also been described. Two similar alcohol dehydrogenases have been purified and characterized in propane-utilizing bacteria.

The genetics of the CMN- complex of bacteria has received scant attention while the OCT plasmid system in *Pseudomonas* sp. was studied in great detail.

CHAPTER 2

MATERIAL AND METHODS

2.1 Media and growth conditions

2.1.1 Organism

The bacterium used in this study was Rhodococcus rhodochrous PNKb1, which was isolated by N.Woods in 1986. This organism was selected from the propane-utilizer culture collection of N.Woods (Woods & Murrell 1989) because it grows rapidly on propane and on most of the potential intermediates in propane metabolism. Rhodococcus rhodochrous PNKb1 is a non-motile, Gram-positive rod. It is catalase-positive, non-spore forming and showed oxidative metabolism of glucose.

2.1.2 Media

Rhodococcus rhodochrous PNKb1 was routinely grown in an ammonium mineral salts medium (AMS) (Whittenbury *et al.*, 1970). The composition of this medium is given in Table 2.1. A solid medium was prepared by the addition of 15 g l^{-1} Difco Bacto-agar to AMS medium. The medium was sterilized by autoclaving at 121°C for 15 minutes. Subsequently, a 1% (v/v) sterilized phosphate stock solution was added to the cool medium to prevent any precipitation of phosphates. Whereas flammable liquid carbon sources such as alcohols were filtered using 0.2 μm sterile solvent resistant filters, propane was considered "sterile". Non-flammable carbon sources like succinate and propanoate were sterilized by autoclaving and added prior to inoculation at the concentrations shown in Table 2.2. Substrates that appear to

Table 2.1. Composition of Ammonium Mineral salts medium and phosphate solution.

Compound	per litre
$Cacl_2$	0.2 g
$MgSO_4.7H_2O$	1.0 g
NH_4Cl	1.0 g
$NaMoO_4.2H_2O$ stock solution	0.5 ml
Fe/EDTA stock solution	0.1 ml
Trace elements stock solution	1.0 ml

$NaMoO_4.2H_2O$ stock solution

$NaMoO_4.2H_2O$ 0.5 g l^{-1}

Fe/EDTA stock solution

Fe/EDTA 3.8 g $100ml^{-1}$

Trace element stock solution

Compound	mg l^{-1}
EDTA	500
$FeSO_4.7H_2O$	200
$ZnSO_4.7H_2O$	10
$MnCl_2.4H_2O$	3.0
Boric acid	30
$CaCl_2.6H_2O$	20
$CuCl_2.2H_2O$	1.0
$NiCl_2.6H_2O$	2.0
$NaMoO_4.2H_2O$	3.0

Phosphate stock solution (0.4 M)

Compound	**$g.l^{-1}$**
$Na_2HPO_4.12H_2O$	71.6
KH_2PO_4	26

Final pH = 6.8

be toxic to the cells at 0.05% (v/v) (propan-2-ol, propanal, acetone, acetol and methyl acetate) were added just before they are exhausted. The wild-type organism was grown on nutrient agar plates to check for purity. Nutrient broth medium was used to obtain mutagenised cells. The above media were prepared according to the manufacturers instructions.

2.1.3 Maintenance of propane cultures

Propane utilizers were sub-cultured monthly to prevent any fungal contamination and to maintain fresh stocks. Stock cultures were streaked onto AMS agar plates and placed in "Gas pack" anaerobic jars. Propane was introduced into anaerobic jars using a football bladder. The concentration of gas was approximately 50% (v/v) propane/air. Containers were incubated in a warmroom at 30°C for 5 days. Propane-grown Rhodococcus rhodochrous PNKb1 and mutants were stored at -20°C in glycerol.

2.1.4 Routine growth on propane and intermediates of propane oxidation

R.rhodochrous PNKb1 was grown on propane and volatile substrates (e.g alcohols) in 250 ml "quick-fit" conical flasks containing 50 ml of AMS medium. Phosphate buffer was added as mentioned previously to the medium. A loopful of propane-grown cells from an agar plate or 0.5 ml of an exponential growth phase culture was used to inoculate the culture. Volatile and aqueous substrates were added to the culture, flasks were sealed with sterilized "Suba-seals" and

Table 2.2. Substrate concentrations used in media

Substrate	Concentration (% v/v except *% w/v)	(mM)
Propane	50% in air	1.42**
Propan-1-ol	0.1	13.4
Propan-2-ol	0.05	6.5
1,2-Propanediol	0.1	13.6
Propanal (Propionaldehyde)	0.05	6.9
Propanone (Acetone)	0.05	6.7
Hydroxyacetone (Acetol)	0.05	7.2
Methyl acetate	0.04	4.99
Propanoate[a]	*0.1	10.4
Methanol	0.1,0.05	12.4,6.2
Methylglyoxal	0.1,0.05	16.4,8.2
Acetate[a]	*0.1,0.035	12.2,4.3
Succinate[a]	*0.1,0.035	7,2.6
Pyruvate[a]	*0.1,0.035	18,6.3

a: sodium salt

**: Solubility in water at 30°C (McAuliffe, 1966).

then the tops were swabbed with 70% (v/v) ethanol. A 100 ml of air was withdrawn from flasks through the "Suba-seal" using a sterile needle and syringe. A 100 ml of propane was injected to give 50% (v/v) propane:air. Then, flasks were incubated in a warmroom (30^{o}C) on an orbital shaker at 200 rpm.

R.rhodochrous PNKb1 was grown in a 2 L LH 500 series fermentor (LH Fermentation, UK). Air and propane were supplied to the 2 L working-volume vessel at 200 and 80 ml min^{-1} respectively. Growth was at a constant pH of 6.8 and at a temperature of 30^{o}C. The oxygen electrode was calibrated using air-saturated medium (100% D.O.T) and then zeroed with nitrogen saturated medium. Then the fermentor was inoculated with 2 X 50ml shake flask cultures in the mid-exponential phase of growth.

2.1.5 Continuous cultures

Continuous cultivation of the cells was attempted using a 2 L LH 500 series fermentor in the same conditions mentioned previously. The medium was sometimes supplemented with 0.035% (w/v) yeast extract . Dilution rates tried were between 0.01 to 0.1 h^{-1}. Cell density was routinely measured at an optical density of OD_{540nm} against a medium blank.

2.1.6 Culture purity

The purity of the cultures was checked regularly. Samples taken from flasks and from the 2 L fermentor were examined

microscopically, serially diluted in AMS medium and plated onto AMS and nutrient agar plates. AMS plates were incubated at 30°C in a propane:air atmosphere as described previously. Nutrient agar plates were incubated at 30°C and 37°C. Colonies from plates were checked morphologically and microscopically.

2.1.7 Growth of mutants in the presence of propane and low concentrations of growth-supporting substrate

The propane oxidation system in the wild-type and NTG-generated propane oxidation mutants was induced by growing cells on succinate at 0.035% (w/v) in the presence of 50% (v/v) propane:air. Under these conditions, the indication of propane-specific polypeptides (section 2.6) and the production of 1,2-epoxypropane from propene by whole cells (section 2.2.4.1) were used to indicate the induction of the propane-oxidizing system. Controls used low concentrations of growth substrates without propane. Similar results were obtained when pyruvate and citrate were used as growth-supporting substrates (Ashraf, 1990).

2.1.8 Cell dry weight determination

Dry weight was estimated by constructing a standard curve of dry weight of cells versus optical density. Dry weight was measured using the filtration method of Gerhardt (1981). Washed samples of cultures at different phases of growth and known optical densities were filtered under vacuum through

preweighed, dried filters of pore size 0.2 μm. Filters and cells were then dried in an oven at 90°C to constant weight.

2.1.9 Light Microscopy

Cultures were examined regularly to check their purity. Slides were prepared by placing a drop of culture on a slide and then pressing it down firmly using a coverslip. The microscope (Kyoma Unilux 11, Japan) was used in phase-contrast mode at a magnification of 1000X.

2.2 Studies using whole cells

2.2.1 Preparation of cell suspensions

Cultures from flasks or fermentors were harvested in the middle of the exponential phase by centrifuging at 20,000 xg for 10 minutes at 4°C. Cell pellets were washed in 20-30 ml of ice-cold 20 mM TRIS-HCl pH 6.8 and centrifuged as before. Then these were resuspended in the same buffer to an E_{540} of between 20 and 50, and this suspension was used for whole cells studies.

2.2.2 Oxygen electrode assays

A Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK) was used to test the ability of R.rhodochrous PNKb1 to oxidize various substrates by measuring the stimulation of oxygen uptake on addition of the substrate to

cell suspensions. Assays were done in a stirred reaction mixture containing 2.9 ml 20 mM phosphate buffer, pH 6.8 at 30^{o}C. An air-saturated buffer was measured in the reaction chamber; the plunger was inserted and the system was allowed to equilibrate. The dissolved oxygen concentration of the air-saturated buffer was calculated using the method of Robinson and Cooper (1970). The output from the O_2 electrode was logged on a chart recorder (Servoscribe 1s). 50 μl of cell suspension were then injected using a syringe and the endogenous rate of oxygen uptake measured. Aqueous substrates were prepared as a 2 mM solution and 50 μl were then injected into the assay mixture to give a final concentration of 33.3 μM (Woods & Murrell, 1989). Propane-saturated solution was prepared by degaussing 5 ml of distiled water under vacuum then passing the contents of a football bladder inflated with propane through the water (Woods, 1988). The final concentration of propane was 23.7 μM [calculated from the data of McAuliffe (1966)]. Substrates which are slightly soluble in water (e.g propane analogues) were prepared as saturated solutions. Endogenous rates of propane oxygen uptake were measured, then 50 μl of propane analogues were added in order to study their effect on propane oxidation. The rates of oxygen uptake were expressed as nmol O_2 min^{-1} mg dry weight of $cells^{-1}$.

2.2.3 Growth of R.rhodochrous PNKb1 on propane analogues

The ability of Rhodococcus to grow on or to oxidize 1-chloro propane, 2-chloro propane and 1,2 di-chloropropane was

tested. These were used as growth substrates at concentration of between 0.025% and 0.05% (v/v). Also, the effect of these compounds on propane oxidation was measured as described above using oxygen electrode technique.

2.2.4 Gas chromatographic assays

A Pye Unicam gas chromatograph (GC) was used to assay the formation of 1,2-epoxy propane from propene, to identify products formed during growth on various substrates and to measure the alcohol(s) extracted from propane-grown cultures. The GC, with a flame ionization detector (FID), was fitted with two 1.5 m x 2.3 mm glass columns, one packed with Poropak Q and the other with mixture of Poropak Q and Poropak N [1:1 (w/w)]. The columns were run isothermally with nitrogen as carrier gas, flow rates and temperature of injector and detector (as mentioned below). An integrator (Hewlett packard) was linked with the GC which had been calibrated with 2 mM standard solutions.

2.2.4.1 Formation of 1,2-epoxy propane from propene

Assays were done in 5ml "Suba-sealed" vials in a total volume of 1 ml. Cell suspension (50 µl) was added to 950 µl of 20 mM TRIS-HCl, pH 6.8. Vials were incubated in a shaking water bath at 200 rpm for 30 seconds then, 3 ml of air were withdrawn from the vial and replaced with 3 ml propene. 5 µl samples were taken every 5 minutes and injected into the Poropak Q column at 180^{o}C while the detector was maintained

at 250°C and the injector at 300°C. N_2 was used as a carrier gas at a flow rate of 30 ml min^{-1}. The rates were expressed as nmol 1,2-epoxy propane formed min^{-1} mg dry weight of $cells^{-1}$.

2.2.4.2 Inhibitor studies

Potential inhibitors were made up as 100 mM solutions [except CO and C_2H_2 which were used as saturated solutions in water having concentrations of 1 mM and 44 mM respectively (Stirling and Dalton, (1978))]. Assays were done, as mentioned above, in 5 ml "Suba-Sealed" vials. Inhibitors were then added to the vials to give a final concentration of 1, 5 or 10mM before adding the substrate. The final concentration of CO and C_2H_2 in the assays was 0.5 & 7.5 mM. Control vials were made without any inhibitors.

2.2.4.3 Analysis of the supernatant from cells grown on propane and potential oxidation intermediates

Rhodococcus was grown in a LH500 fermentor on propane as the sole source of carbon and energy and on potential intermediates in the pathway of propane. Samples were harvested at various phases of growth and supernatants were analysed using the GC. 5 µl samples of the supernatants were injected into two different columns; Poropak Q at 180°C and Poropak Q/N at 150°C (GC conditions as mentioned in section 2.2.4.1 & 2.2.4.4).

2.2.4.4 Excretion of alcohol(s) from propane-grown PNKb1 in the presence of various inhibitors

Assays were performed in 10 ml "Suba-sealed" vials in a total volume of 2 ml. Various concentrations of inhibitors that inhibit the alcohol dehydrogenas(s) were mixed with AMS medium in sealed vials. The inhibitors used were: phosphate, phosphate in 2 M sodium chloride, ammonium chloride and EDTA. The final concentration of the above inhibitors in the assays were 50 and 100 mM except for ammonium chloride (10 & 25 mM). All vials were incubated in a shaking water bath at 30^{o}C for 1 minute. 100 μl of cell suspension were added to give a final OD_{540} between 5-10. Then, 4ml of air were withdrawn from vials and 4 ml of propane were injected. Vials were reincubated and 5 μl sample was taken every 30 minutes and injected into the GC fitted with Poropak Q/N [1:1 (w/w)] glass column at 150^{o}C with nitrogen (30 ml min^{-1}) as a carrier gas. The injector and the detector were maintained at 300^{o}C and 250^{o}C respectively. Retention times (minutes) of all the possible volatile products of the propane oxidation pathway are shown in table 2.3.

2.3 Studies using cell-free extracts

2.3.1 Cell breakage techniques

Three breakage methods were used to obtain satisfactory cell-free extracts. Cell suspensions were prepared as described in section 2.2.1. and used as follows:

Table 2.3. Retention times (min) of all detectable potential intermediates in the propane oxidation pathway

	Column type	
solvent	**Poropak Q**	**Poropak Q/N**
Methanol	0.62	1.55
Propan-1-ol	3.27	12.7
Propan-2-ol	2.4	9.3
Propanal	1.87	7.5
1,2-Propanediol	12.56	N.D
Acetone	2.22	8.00
Acetol (1-Hydroxy acetone)	8.15	N.D
Methyl acetate	8.16	6.4

N.D : Not determined

a) French pressing: cell suspensions were passed (three passages) through a French pressure cell (Aminco, Silver Spring, Maryland, USA) at a pressure of 138 MPa. The pressure cell was cooled to 4°C and extracts collected on ice in precooled Universals.

b) Sonication: Cell suspensions were subjected to seven bursts of ultrasound using a sonicator (MSE, Crawley, Sussex, UK) at maximum amplitude (24 microns),each lasting 30 seconds. The cells were kept on ice between bursts.

c) Cell disintegration for PAGE analysis: Cell suspensions were boiled for 5, 10 and 15 minutes in 4% (v/v) sodium dodecyl sulphate (SDS). Supernatant was used for SDS-PAGE.

2.3.2 Preparation of cell-free extracts

After preparation of cell suspensions (section 2.2.1), cells were broken using one of the above techniques. Unbroken cells were removed by centrifugation at 20,000 xg for 5 minutes at 4°C. A second spin was required to separate soluble and membrane bound proteins. Crude extracts were centrifuged at 48,000 xg for 30 minutes at 4°C. The supernatant was then taken as the soluble fraction and the pellets were resuspended in 20 mM TRIS-HCl pH, 6.8 as the particulate fraction. The protein content of extracts was

determined using the method of Bradford (1976) and following the manufacturers (Bio Rad) instructions related to the standard protein microassay. A standard curve was prepared using bovine serum albumin.

2.4 Enzyme assays

2.4.1 Formation of 1,2-epoxy propane from propene

The formation of 1,2-epoxy propane was detected using GC. Assays were performed in 2 ml GC crimp vials in a total volume of 0.25 ml. The buffer used was 20 mM TRIS-HCl, pH 6.8 together with 1-5 mg of protein from a cell-free extract. A vial was sealed and preincubated at 30°C in a shaking water-bath (250 rpm) for 30 seconds. 1 µmol NADH were added and 0.9 ml of air was removed from the vial and replaced with 0.9 ml propene. The vial was then re-incubated in the water-bath. 5 µl samples were removed every 5 minutes and injected into a GC fitted with Poropak Q/N. The operating conditions of the GC were as described in section 2.2.4.1.

2.4.2 Alcohol dehydrogenase

The NAD $(P)^+$-dependent activity was measured spectrophotometrically. A Pye Unicam sp 1800 spectrophotometer monitored the alcohol-dependent change in absorbance at 340nm due to the formation of NAD(P)H. Assays were done in 1.5 ml cuvettes in a total volume of 1 ml.

20 mM TRIS-NaOH, pH 10, 0.2 μmol NAD^+ and a sufficient amount of protein (0.1-1.0 mg), which gave a linear rate for more than 5 min, were placed in a cuvette. The cuvette was allowed to equilibrate for 1 min at 30°C before adding the substrate. To initiate the reaction, 10 μmol of alcohol(s) were added to the cuvette. The change in absorbance at 340 nm was followed for 5-10 min.

2.4.3 Acetol and acetone monoxygenase

The acetol and acetone monoxygenase activity was measured using spectrophotometric and oxygen electrode assays:

(1) Oxygen electrode assay: This was measured as the substrate-dependent stimulation of oxygen uptake in a Clarke-type oxygen electrode. This technique is based on the method of Hartmans & de Bont (1986). A sufficient amount of 20 mM TRIS-HCl, pH 6.8, to give a final volume of 3 ml was equilibrated at 30°C in the oxygen electrode. Cell-free extract was added to give 0.1-1.0 mg of protein and the endogenous uptake was recorded. 1 μmol of NAD(P)H was added and the change in rate measured. 8 μmol of sustrate was subsequently added and any further change in rate recorded.

(2) Spectrophotometric assay: This was measured spectrophotometrically using a Beckman DU-70 spectrophotometer (Beckman instruments Ltd., High Wycombe, U.K) by monitoring the change in absorbance at 340 nm due to the reduction of NADH to NAD^+. Assays were done in a total

volume of 1 ml. The cuvettes which contained 20 mM TRIS-HCl, pH 6.8, 0.2 μmol of NADH and 1-1.5 mg protein were allowed to equilibrate at 30°C for 1 min. The reaction was initiated by the addition of 5 μmol acetol or acetone. The change of absorbance at 340 nm was followed for 5 min.

2.5 Partial purification of acetol monooxygenase

Several techniques were used in an attempt to isolate acetol monooxygenase from cell-free extracts of acetol-grown R.rhodochrous PNKb1. All procedures were carried out at 4°C.

Step 1 Ion-exchange chromatography:

a) 10 mg of protein from a cell-free extract was loaded onto a 10 cm x 10 mm FPLC column packed with Mono Q. The column was washed with 30 ml of 20 mM Tris-HCl buffer pH 7.0. The column was operated at a flow rate of 0.5 ml min^{-1} for 20 min. and then the flow rate was increased to 1.0 ml min^{-1}. The column was eluted batchwise with 0%, 10%, 20%, 50% and 100% 1 M NaCl. 4 ml fractions were collected and acetol monooxygenase activity was assayed. Fractions were also assayed for the ability to epoxide propene to 1,2-epoxypropane (see section 2.4.1).

b) 500 mg of protein from a cell-free extract was loaded onto a 26 cm x 10 mm FPLC column packed with DEAE-Sepharose. The column was washed with 20 mM Tris-HCl buffer pH 7.0. The column operated at a flow rate of 8 ml min^{-1}. The column was eluted batchwise with 0%, 40%, 50% and 100% 1 M NaCl. Fractions were collected and acetol monooxygenase activity

was assayed. Fractions were also assayed for the ability to convert propene to 1,2-epoxypropane. (see section 2.4.1). Those fractions showing significant acetol monooxygenase activity were concentrated in an Amicon model (8050) ultrafiltration unit fitted with a PM 10 ultrafiltration membrane to give a final volume of 5 ml.

Step 2 Gel filtration: the concentrated pooled fractions from step 1 were applied to a 60 cm x 26 mm FPLC column packed with Superdex 200 (S-200). The column was washed with 20 mM Tris-HCl buffer pH 7.0. The column operated at a flow rate of 3 ml min^{-1} and fractions of 3 ml volume were collected. Pooled fraction were concentrated, as in step 1, to a final volume of 2 ml.

Step 3 Ion-exchange chromatography:
The concentrated fractions from step 2 were applied to a column Mono Q. The column was eluted as described in step 1. Fractions contained acetol monooxygenase activity were then concentrated, as in step 1, to a final volume of 2 ml and then stored at -70°C.

2.6 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Woods & Murrell (1989). Single percentage gels of 10.5% acrylamide (w/v) or 10 to 30% (w/v) gradient gels were used. 50-100 µg protein of cell-free extracts were loaded onto each track. Gels were stained using Coomassie

Blue R in acetic acid and methanol and then destained in the same solvent.

2.7 Western-Blotting

This was based essentially on the method described by Ashraf & Murrell (1992). Proteins separated by SDS-PAGE were transfered to a nitrocellulose filter using a Trans-Blot (Bio-Rad) system. The filter was stained in Ponceau S to visualize proteins. After washing treatments, the primary antibody was added. Unbound antibodies were removed by washing the filter in TBS. Secondary antibody (peroxidase conjugated goat anti-rabbit IgG) was allowed to bind with the primary antibody. After the filter was washed, it was stained using 4-chloronaphthol as a colour reaction agent.

2.8 Mutagenesis

In order to obtain point, double and deletion mutants, R.rhodochrous PNKb1-Str^{r}_{20} was subjected to two types of chemical mutagenesis.

2.8.1 N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenesis

NTG is a potent mutagen and carcinogen. It must be handled with caution. A stock solution was prepared to give a final concentration of 1000 $\mu g\ ml^{-1}$ NTG in distilled water. Stocks were sterilized using a 0.22 μm filter and stored at -20°C until required.

R.rhodochrous PNKb1-Str^r_{20} was grown overnight in nutrient broth to obtain an optical density of 0.6 at 540 nm. Cultures were incubated with NTG at 150 $\mu g\ ml^{-1}$ at 30°C in a shaking water-bath for 10-12 min. This gave 50% "kill" (Ashraf, 1990). A 10 ml sample was then taken and washed twice with ice-cold sterilized AMS medium. Pellets were resuspended in AMS medium and incubated overnight at 30°C in a 50 ml nutrient broth medium to allow for "recovery" time. A sample was serially diluted in AMS medium and then plated onto nutrient agar plates. Individual colonies were replica-plated onto AMS plus 0.1% (w/v) succinate (master plate) and AMS/propane plates for selection of propane- deficient mutants. To select mutants defective in particular steps of propane metabolism, colonies from the master plates were plated onto AMS plates plus potential intermediates of propane oxidation . After that plates were placed in a Tupperware box and volatile intermediates (e.g propan-2-ol, acetone ...etc) were spotted onto filter papers in a Petri-dish. All boxes were sealed with plastic tape and incubated at 30°C for various times depending on the carbon source deficiency being screened. Finally, individual mutants were tested for growth on various liquid medium of potential intermediates in the propane pathway.

2.8.2 DEO and DEB-mutagenesis

This was carried out using methods similar to those above. 20 µl of 1,2,3,4 di-epoxybutane (DEB) or 10 µl of 1,2,7,8 di-epoxyoctane (DEO) was added to 10 ml culture. 5 µml

samples were taken at intervals, washed in AMS medium and plated to give viable counts. The results were used to construct a "kill curve".

To generate mutants, cultures of DEB were incubated for 8.5 and 32 min to give 50% and 95% "kill" respectively.

2.8.3 Penicillin enrichment

NTG-mutagenized cells were washed and incubated in an AMS liquid medium supplemented with a carbon source required for selecting a specific mutant. After 10,000 U of penicillin-G/ml were added, the incubation was continued for 60-90 min and the culture was washed 3 times by centrifugation at 5,000 xg for 5 min to remove penicillin-G. Pellets were resuspended in AMS medium and incubated overnight in 50 ml of nutrient broth at 30°C to allow for "recovery" time. The culture was resuspended in 10 ml in AMS medium and then serially diluted onto nutrient agar plates. Individual colonies were plated as previously described (see section 2.8.1).

2.9 Transmission electron microscopy

This was based on the method of Spurr (1969). Propane-grown R.rhodochrous PNKb1 was harvested in the middle of the logarithmic phase and washed twice in 20 mM TRIS-HCl, pH 6.8. Pellets were washed in (1:10) Kellenberger fixative: distiled water and then resuspended in 5 ml Kellenberger

fixative. Following that, 0.5 ml of fresh tryptone medium was added and then cells were transfered to a glass stoppered bottle which was left overnight in the fridge. The contents of the bottle were washed in 10 ml and 5 ml Kellenberger buffer respectively. Pellets were mixed with a minimum amount of Bacto agar and left on a Petri-dish to set. Small squares "$1mm^2$" were made from the agar and kept in 10 ml washing fluid for 2-3 hours in a vial and then washed twice in Kellenberger buffer. Specimens were dehydrated by soaking in a graded series of ethanol solutions (30,50,70,90%), for 30 min, and finally soaking in 100% ethanol for 60 min. After the last treatment, an equal quantity of embedding medium was added to that of the dehydrating fluid. The vial was mixed and left for 30 minutes. Then another equal quantity of embedding medium was added to the mixture and the vial was left for 30 minutes. The vial was drained of all the mixture and 10 ml of embedding medium was added. Specimens were then in 100% embedding medium. Vials were left rotating overnight in a cold-room at 2rpm. Specimens were transfered into "oven-dry gelatin" capsules and the capsules were filled up with embedding medium. These were left to polymerize in an oven at $70^{o}C$ for 8 hours. The specimens were then ready for sectioning. Sections were prepared with an ultra microtome (Reichert) using a diamond knife. Thereafter, sections were examined using a JEM-100S electron microscope at a magnification of 10,000-25,000.

2.10 Photography

Polyacrylamide gels and nitrocellulose filters were photographed from above using a Canon camera with Agfa Otho film.

2.11 Chemicals

Most compounds, substrates, media, inhibitors, cofactors, etc, were obtained from the following manufacturers: Aldrich Chemicals Co Ltd., Gillingham, Kent, UK; Sigma (London) Chemicals Co Ltd., Poole, Dorest, UK; BDH Chemicals Ltd., Poole, Dorest, UK; Kodak Laboratory Chemicals, Queensferry, Clwyd, UK; Bio-Rad Laboratory Ltd., Hemel Hempstead, Hertfordshire, UK; Fisons Scientific Apparatus, Loughborough, Leics.,UK; British Oxygen Co Ltd., London, UK.

CHAPTER 3

GROWTH ON PROPANE, POTENTIAL INTERMEDIATES IN PROPANE METABOLISM AND SUBSTITUTED PROPANE

3.1 Introduction

Owing to its ability to grow on propane, as source of carbon and energy, Rhodococcus rhodochrous PNKb1 is an interesting organism to study. It also grows on most of the potential intermediates in propane metabolism except methyl acetate, methanol and formaldehyde (Woods, 1988). Recently, it has been found that R.rhodochrous PNKb1 is able to grow on methyl acetate only at low concentrations 0.04% (v/v), see section 3.6. Propane-grown cells synthesized three propane-specific polypeptides of approximately 69, 59 and 57 kDa (Woods, 1988). Similar propane-specific polypeptides were observed with both propane and acetol-grown cells (Ashraf, 1990).

NAD^+-dependent secondary alcohol dehydrogenase has been purified from propane-grown cells (Ashraf and Murrell, 1990). Antibody was raised and used in Western-blot analysis of enzyme induction after growth on propane and oxidation intermediates. This enzyme was only synthesized after growth on propane and some of the subterminal intermediates. It was also synthesized in other propane-utilizing bacteria during growth on propane (Ashraf, 1990).

Both NAD^+-dependent primary and secondary alcohol dehydrogenase activities were detected in the soluble fraction of cell-free extracts of propane-grown cells (Ashraf, 1990). However, the activity of 1,2-propanediol (1,2-PDL) alcohol dehydrogenase was not tested. In this

study, the specific activity of propan-1-ol, propan-2-ol and 1,2-PDL alcohol dehydrogenase(s) was analysed in order to gain a better understanding of the alcohol dehydrogenase systems that occur in the metabolism of propane and 1,2-PDL pathways.

It has been found that methanol is accumulated from methane oxidation in response to the addition of sodium chloride, potassium chloride or phosphate (100 mM) to whole cells of methane-grown Methylosinus trichosporium (Mountfort *et al.*, 1990). Following an extra addition of 40 mM sodium formate at the end of 6 hours cultivation, the accumulation of methanol was restored at the highest level (Mehta *et al.*, 1990). However, Mountfort *et al.* observed that the addition of EDTA resulted in an accumulation of alcohol from the oxidation of ethane and propane. Accumulation of alcohols from whole cells of propane-grown PNKb1 could be possible using some of the potential inhibitors described earlier. This should show whether terminal or subterminal oxidation is the major route for propane metabolism.

Excretion of potential products during growth on propane and oxidation intermediates in propane metabolism has not been investigated in PNKb1 previously. Therefore, culture supernatants were analysed in order to allow detection of any products that are synthesized during growth on various substrates. This type of experiment could demonstrate different steps of the oxidation sequences of the pathways for terminal and the subterminal oxidation of propane.

3.2 SDS-PAGE of protein profiles from propane and potential oxidation intermediates-grown cells.

The protein profiles of soluble cell-free extracts from PNKb1 after growth on propane and potential oxidation intermediates are shown in Fig. 3.1. Similar specific polypeptides of approximately 69, 59 and 57 kDa that have been suggested to be components of the propane-specific oxygenase (Woods, 1988) were looked for in this study with propane, acetol, acetone, propan-2-ol and 1,2-PDL-grown cells. However, the propane-specific polypeptides were only synthesized after growth on propane and acetol (Ashraf, 1990). These differences in terms of the induction of polypeptides could be due to toughness of the cell wall breakage when cells were grown on propan-2-ol, acetone or 1,2-PDL. Results from this study showed that the amount of protein per ml in cell-free extracts of propane-grown cells is greater than that obtained from propan-2-ol, acetone or 1,2-PDL-grown cells. In addition, Ashraf (1990) reported that low amounts of the propane-specific polypeptides were observed with propan-2-ol-grown cells. Nevertheless, only one of these polypeptides of 68 kDa was not synthesized with 1,2-PDL-grown cells, whereas the others were induced as, was found in propane and acetol-grown cells (Ashraf, 1990). In this study, the synthesise of the propane-specific polypeptides in cell-free extracts of 1,2-propanediol-grown cells suggests a relationship between the metabolism of these two compounds. However, an additional "specific" polypeptide of 36 kDa was also observed. This could be

Figure 3.1 SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane and various potential oxidation intermediates.

Track	Growth substrate
1	(Molecular weight markers)
2	Propane
3	Propan-1-ol
4	Propan-2-ol
5	1,2-Propanediol
6	Acetol
7	Acetone
8	Succinate
9	Methyl acetate

100 μg protein in each track

N.B the same gel was duplicated to be use for Western-blot, see Fig. 3.2.

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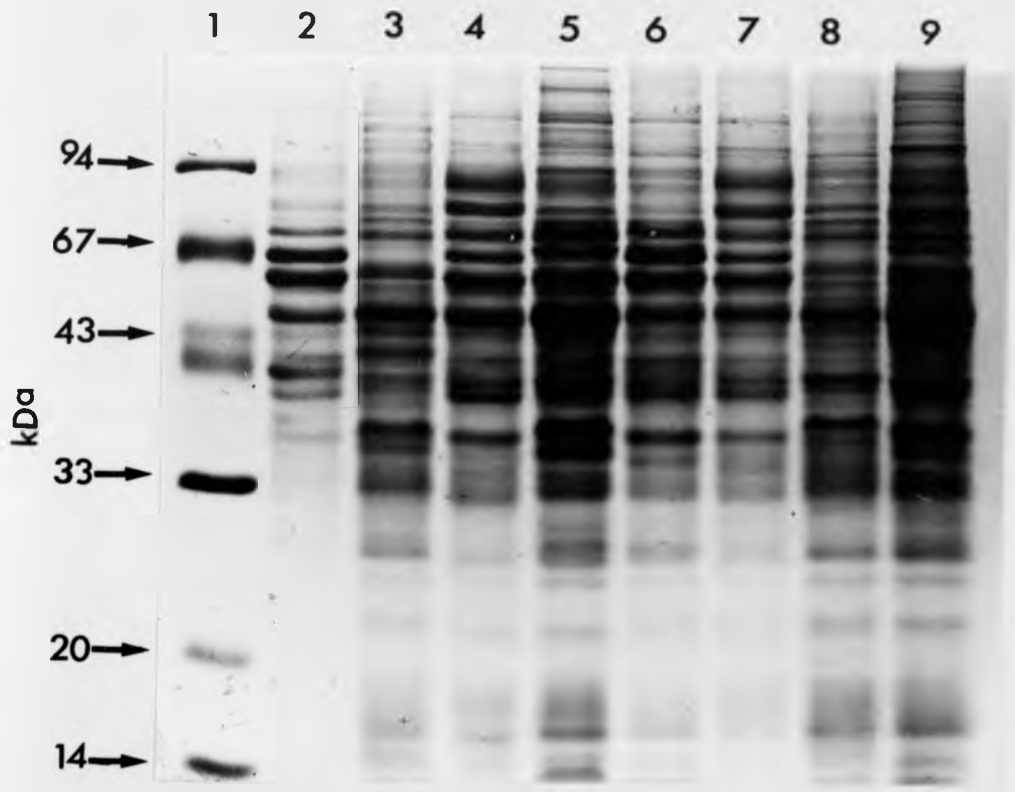

required for the metabolism of 1,2-PDL. Simultaneous adaptation studies shows that 1,2-propanediol was oxidized by propane-grown PNKb1. Also, 1,2-propanediol-grown cells oxidized propane (Ashraf, 1990). These results might suggest the involvement of a novel propane dioxygenase which oxidized propane via 1,2-propanediol and then to acetol (see Fig. 3.10).

Acetone-grown cells were also shown to the synthesize the propane-specific polypeptides (Fig. 3.1). However, additional "specific" polypeptides of 92 and 84 kDa were also observed in cell-free extracts of acetone-grown cells. The same result was obtained several times using different samples of cell-free extracts. These unique polypeptides may be required for the metabolism of acetone.

Protein profiles of cell-free extracts after growth on methyl acetate were completely different from those obtained from propane-grown cells (Fig. 3.1). There were no similarities in terms of the induction of the specific polypeptides after growth on methyl acetate compared with propane. Also, many distinct polypeptides were present in cell-free extracts of methyl acetate-grown cells while they were absent in extracts from propane-grown cells. This indicates that methyl acetate is metabolized by a different, separate pathway (for more evidence see section 3.6).

3.3 Synthesis of the NAD^+-dependent secondary alcohol dehydrogenase

Ashraf and Murrell (1990) reported that the NAD^+-linked secondary alcohol dehydrogenase was only induced after growth on propane, on some of the intermediates of the subterminal pathway (e.g propan-2-ol and acetol but not acetone) and 1,2-PDL. This suggested to them that a common regulatory system for the metabolism of propane and acetol exists in PNKb1 whereas a different pathway might be involved in the metabolism of acetone. Nevertheless, in this study the synthesis of the secondary alcohol dehydrogenase was also observed after growth on acetone and 1,2-PDL in lower amounts than found in propane, propan-2-ol and acetol-grown cells (Fig. 3.2). The purified secondary alcohol dehydrogenase oxidized 1,2-propanediol at a low rate when compared with propan-1-ol and propan-2-ol (Ashraf and Murrell, 1990). In addition, 1,2-PDL-grown cells exhibited a low rate of secondary alcohol dehydrogenase when compared with propane or propan-2-ol-grown PNKb1 (see Table 3.1). These results, along with the synthesis of secondary alcohol dehydrogenase after growth on 1,2-PDL, confirmed the previous suggestion that there might a link between the metabolism of propane and 1,2-PDL (see section 3.2).

Figure 3.2 Corresponding Western-blot analysis using antibodies against a purified NAD^+-dependent alcohol dehydrogenase.

Track	Growth substrate
1	(Molecular weight markers)
2	Propane
3	Propan-1-ol
4	Propan-2-ol
5	1,2-Propanediol
6	Acetol
7	Acetone
8	Succinate
9	Methyl acetate

100 μg protein in each track

N.B this gel was a duplicate of that shown in Fig. 3.1.

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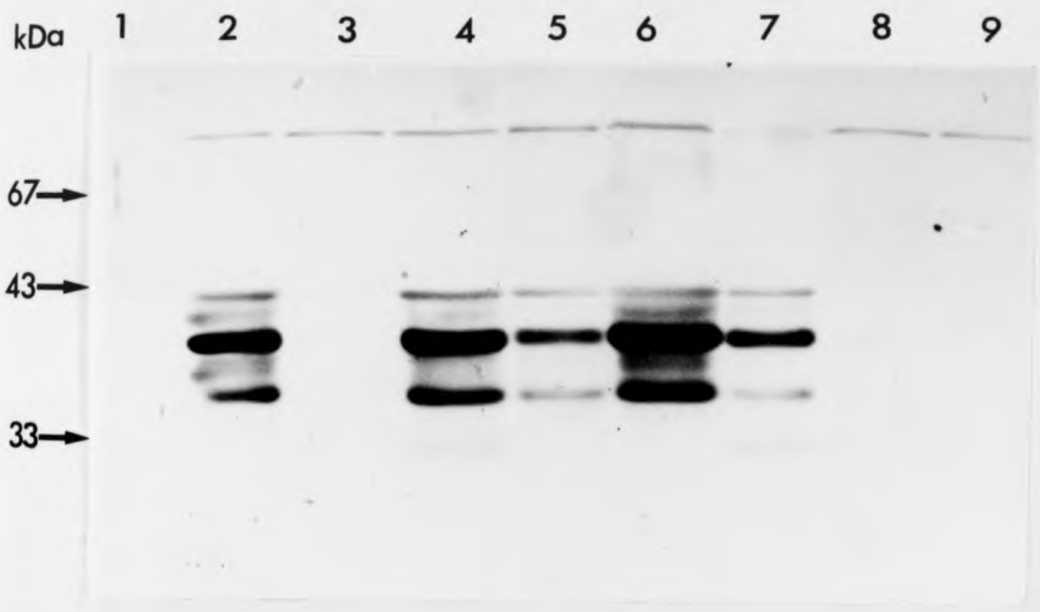


hat shown in

Table 3.1 Specific activities of alcohol dehydrogenases in cell-free extracts of propane, propan-1-ol, propan-2-ol and 1,2-propanediol-grown R.rhodochrous PNKb1.

Enzyme Activity	Cells grown on: Propane	Propan-1-ol	Propan-2-ol	1,2-PDL
	Specific Activity (units)[a]			
Propan-1-ol dehydrogenase	35	12	14	11
Propan-2-ol dehydrogenase	170	31	72	22
1,2-Propandiol dehydrogenase	4	9	2.5	24

[a] 1 unit = 1 nmol NAD^+ reduced min^{-1} mg $protein^{-1}$

3.4 Alcohol dehydrogenase(s) activities from cells grown on propane and intermediates of propane oxidation

The specific activities of NAD^+-dependent primary, secondary and diol dehydrogenase(s) are shown in Table 3.1. Cells grown on propane or propan-2-ol, but not propan-1-ol or 1,2-PDL, were found to have elevated levels of NAD^+-dependent secondary alcohol dehydrogenase activity. In contrast, propan-1-ol, propan-2-ol and 1,2-PDL-grown cells showed lower levels of primary alcohol dehydrogenase activity when compared with propane-grown cells. However, very low rates of diol dehydrogenase activity were observed after growth on propane and propan-2-ol whereas propan-1-ol and 1,2-PDL-grown cells showed a relatively higher rate. These results suggest that there might be more than one NAD^+-dependent alcohol dehydrogenases involved in the metabolism of propane. Purification of diol dehydrogenase from propane-grown cells will indeed confirm whether 1,2-PDL is involved in the metabolism of propane or not. The existence of more than one alcohol dehydrogenase involved in alkane metabolism is not unique. For instance work carried out by Beers (1988) on butane-grown P.butanovora, showed that four alcohol dehydrogenases were involved in the metabolism of butane (section 1.4.2.2).

3.5 Excretion of alcohols due to the inhibition of alcohol dehydrogenase(s) during growth on propane

Attempts to detect the accumulation of alcohols in cell-free extracts of propane-grown cells were unsuccessful (Woods, 1988). Therefore, excretion of propan-1-ol, propan-2-ol and 1,2-PDL from resting cells of propane-grown PNKb1 was tested using inhibitor studies. Various potential inhibitors (e.g ammonium chloride, phosphate, phosphate in 2 M sodium chloride and EDTA) were used to inhibit the alcohol dehydrogenase(s). Propan-2-ol was the only alcohol produced from resting cells of propane-grown PNKb1 using G.C. No other products or potential intermediates were detected. Control experiments showed no accumulation of any products. Table 3.2 shows the accumulation of propan-2-ol from resting cells of propane-grown PNKb1 using various inhibitors. A detectable quantity of propan-2-ol was excreted by resting whole cells after growth on propane at various concentration of ammonium chloride and phosphate. Addition of ammonium chloride to cell suspension of propane-grown PNKb1 resulted in the accumulation of propan-2-ol; and the most effective concentration was 140 μM. Propan-2-ol was excreted after 30 minutes incubation and then increased gradually until it reached the highest concentration at 7 hours. Thereafter, it began to decline again. However, results from Table 3.2 show that NH_4Cl was more effective than phosphate in leading to the accumulation of propan-2-ol during growth on propane. Stephens (1986) reported that ammonium chloride inhibited alcohol oxidation in strain EI2 after growth on propane

Table 3.2 The effect of various inhibitors on the formation of propan-2-ol from whole cells of propane-grown *R.rhodochrous* PNKb1

Inhibitor	Concentration (mM)	Concentration of propan-2-ol (μM) Time (hours) 0	0.30	1	2	3	4	5	7	9	21
None	-	0	0	0	0	0	0	0	0	0	0
NH_4Cl	10	0	20	20	ND	32	ND	40	40	42	46
	25	0	10	20	40	50	ND	70	140	130	70
Phosphate	50	0	10	ND	20	ND	30	40	ND	35	40
	100	0	0	ND	10	ND	20	37	ND	40	40
Phosphate	50	0	0	0	ND	0	ND	ND	0	0	0
in 2 M NaCl	100	0	0	0	ND	0	ND	ND	0	0	0
EDTA	50	0	ND	0	0	ND	ND	0	ND	0	0
	100	0	ND	0	0	ND	ND	0	ND	0	0

ND = not determined

resulting in the accumulation of both propan-1-ol and propan-2-ol.

With phosphate in 2 M NaCl, there was no accumulation of alcohols were detected from resting cells of propane-grown PNKb1. Mountfort *et al.*, 1990 reported that 0.2 M NaCl did not cause accumulation of alcohol during ethane and propane oxidation, but the salt caused a transient accumulation of methanol with methane-grown Methylosinus trichosporium. Addition of EDTA however resulted in accumulation of alcohol from the oxidation of methane, ethane and propane, and the most effective concentration was 2 mM. They also found that alcohol accumulation was further improved by addition of H_2. They gave no clear-cut explanation why H_2 improved alcohol accumulation in the presence of EDTA. Yet, in contrast with the results obtained from this work, no effect was observed with EDTA in accumulating alcohols during the incubation of resting cells of propane-grown R.rhodochrous PNKb1. This could be due to the high concentration used in this study. Mountfort *et al.* (1990) found that increasing the EDTA concentration beyond 2 mM resulted in the decline of the rate of alkane oxidation along with a decrease in product formation. It would be interesting to test the effect of EDTA at a lower concentration.

3.6 Excretion of some potential intermediates during growth on propane and oxidation intermediates.

R.rhodochrous PNKb1 was grown on propane and various potential intermediates in the metabolism of propane. Samples were taken at various times through the growth curve and cells removed by centrifugation. Culture supernatants were analyzed using G.C.
The accumulation of various potential intermediates during growth on propane and other growth substrates are summarized in Table 3.3. A considerable amount of acetone was accumulated during growth on propan-2-ol. Stephens and Dalton (1986) detected a significant amount of acetone in the supernatant of propane-grown Arthrobacter sp. Similar results have been reported in Mycobacterium smegmatis 422 (Lukins and Foster, 1963) (section 1.3.3.5).

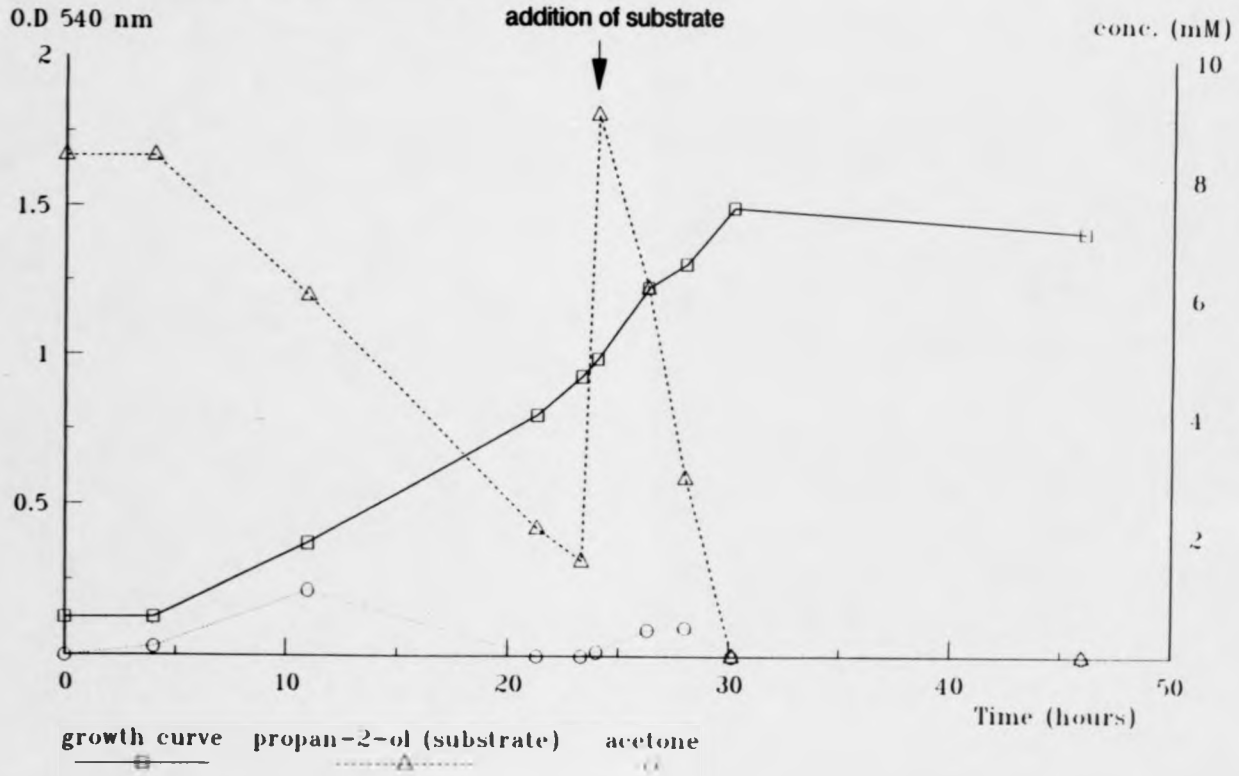
Figure 3.3 shows a transient excretion of acetone during growth on propan-2-ol. Woods and Murrell (1989) reported that Propan-2-ol is toxic to the cells at a concentration of 0.1% (v/v). Therefore, the medium was supplemented with propan-2-ol at a maximum concentration of 0.05% (v/v), and an additional amount of the substrate was added just before exhausted. Acetone was accumulated at low concentration (<1 mM) in the medium after growth on propan-2-ol. The accumulation of acetone during growth on propan-2-ol could be due to a low rate of acetone oxidation. Woods (1988) found that the rate of propan-2-ol oxygen uptake in propan-2-ol-grown PNKb1 was tow-fold that obtained with acetone as

Table 3.3 Accumulation of various potential intermediates during growth on propane and other propane-oxidation pathway

Growth substrate	Products formed	conc. (mM)
Propane	N.D	N.D
Propan-2-ol	Acetone	1.1
Acetone	N.D	N.D
Acetol	N.D	N.D
1,2-propanediol	Acetol	0.29
Methyl acetate	Methanol	68

N.D = None detected

Figure 3.3 Excretion of actone from propan-2-ol-grown R.rhodochrous PNKb1.



a growth substrate. This indicate that the oxidation of acetone is lower than that of propan-2-ol resulting of an accumulation of acetone. The later was only metabolized when the medium was lacking in the substrate propan-2-ol. A general conclusion can be drown from the above discussions that acetone could be an intermediate in the metabolism of propane (For further evidences, see section 5.3.2). Taylor *et al.*, (1980) suggested that propan-2-ol can be metabolized to acetone and then to acetol (section 1.3.3.5).

R.rhodochrous PNKb1 was also grown on 1,2-propanediol and the culture supernatant was analyzed. Although 1,2-PDL was not reported to be an intermediate in the metabolism of propane, it potentially could be for the following reasons:

a) 1,2-PDL is reported to be metabolized via acetol (Hartmans and de Bont, 1986).

b) It has been postulated that a relationship between the metabolism of propane, acetol and 1,2-PDL might be involved due to the synthesis of the secondary alcohol dehydrogenase after growth on the above compounds (Ashraf, 1990).

c) 1,2-PDL-grown cells synthesize the propane-specific polypeptides, see section 3.2. In addition, propane is oxidized by 1,2-PDL-grown cells (Ashraf, 1990).

d) alcA$^-$ (propan-1-ol$^-$), alcB$^-$ (propan-2-ol$^-$) and alcAB$^-$ (propan-1-ol$^-$ and propan-2-ol$^-$) mutants still grow on 1,2-PDL (see section 5.1).

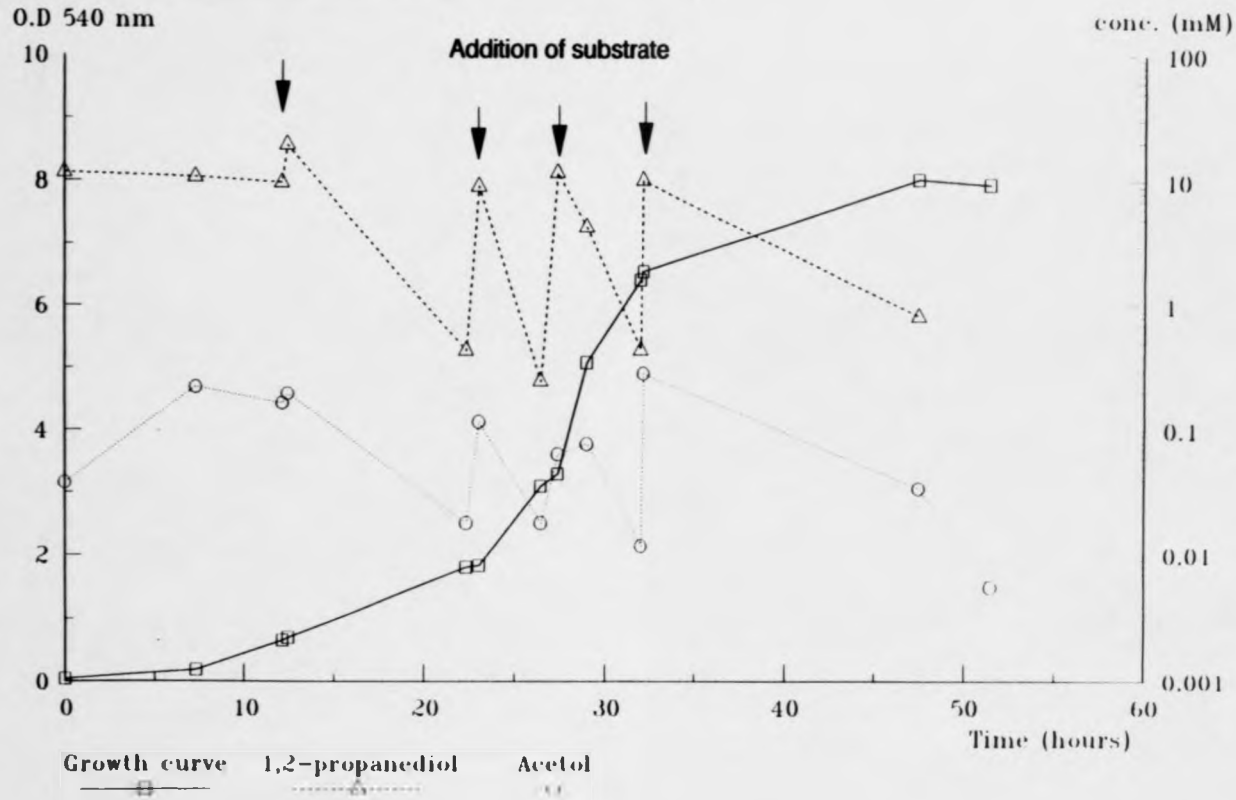
e) Alk$^-$ (propane$^-$) still utilize propan-1-ol, propan-2-ol and 1,2-propanediol as growth substrate, indicating that the

above compounds are involve in the metabolism of propane (see section 5.1).

Figure 3.4 shows the excretion of acetol from 1,2-PDL-grown cells. Acetol was the only intermediate detected during growth on 1,2-PDL. The accumulation of acetol during growth on 1,2-PDL was only transient. It was metabolized when the medium was lacking the substrate (1,2-PDL), indicating that acetol could be an intermediate in 1,2-PDL pathway. The accumulation of acetol could also suggest that 1,2-PDL was probably metabolized to propanal. de Bont *et al.* (1982) reported that Nocardia sp. could metabolize 1,2-PDL to propanal, propionate and then to succinate. Detection of diol dehydratase activity in propane and 1,2-PDL-grown cells would confirm this suggestion. Jetter (1990) detected high levels of diol dehydratase during growth on 1,2-PDL (see section 1.3.3.4).

Recently, high optical density was observed when cells were grown on 1,2-PDL. The results obtained from this work showed that PNKb1 could grow on 1,2-PDL up to 5% (v/v) and the $O.D_{540}$ reached above 20. However, the maximum optical density obtained from R.rhodochrous PNKb1 after growth on propane or potential oxidation intermediates was a round 1.6. This phenomenon could be use to detect a high concentration of acetol excreted from 1,2-propanediol-grown cells.

Figure 3.4 Excretion of acetol from 1,2-propane-diol-grown R.rhodochrous PNKb1.



R.rhodochrous PNKb1 was also grown on acetone and acetol and culture supernatants were analyzed. No potential intermediates were detected in the supernatant when cells were grown on the above substrates (see Fig. 3.5 & Fig. 3.6 respectively). No product whatsoever was also detected from propane-grown cells. The reason behind this is not known. It could be due to the rapid oxidation of the potential intermediate in the terminal and subterminal oxidation of propane metabolism. Woods (1988) failed to detect any accumulation of alcohol(s) in cell-free extracts of propane-grown cells. Stephens (1983) reported that the accumulation of acetone in the growth medium of various strains of propane utilizers does not provide good evidence for the involvement of acetone in the metabolism of propane.

It has been suggested that propan-2-ol was metabolized via methyl acetate (Markovetz, 1972). However, no clear evidence was proposed in the literature that methyl acetate could be an intermediate of propane oxidation. Methyl acetate is highly volatile, toxic and unstable under normal conditions and data on its microbial degradation are not relatively available. One documented paper described the isolation of two Pseudomonas sp. that could utilize methyl acetate at a concentration of 0.2% (v/v) resulting in an accumulation of methanol (Rakov *et al.*, 1990). Nevertheless, Woods and Murrell (1989) reported that methyl acetate did not support growth at 0.1 or 0.05% (v/v). In this study it has been observed that PNKb1 is capable of growth on methyl acetate at concentration of 0.04% (v/v) (Fig. 3.7). No such growth

Figure 3.5 Analysis of the supernatant from acetone-grown R.rhodochrous PNKb1

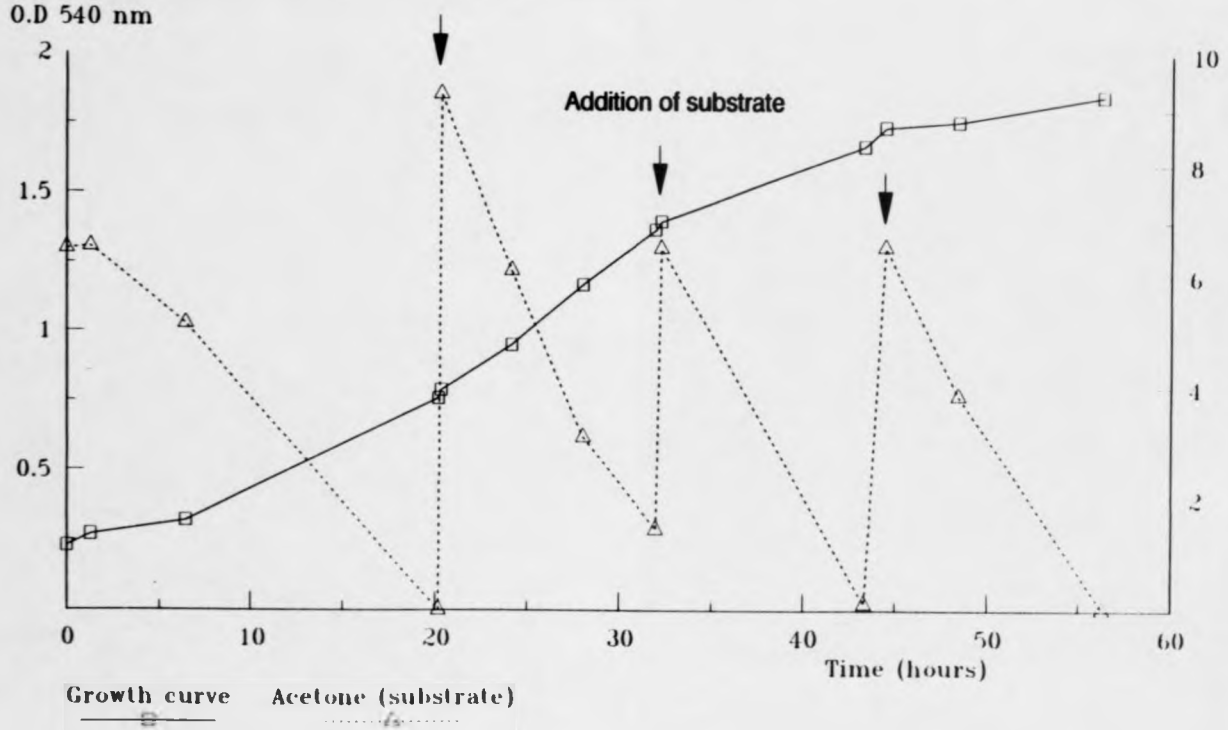


Figure 3.6 Analysis of the supernatant from acetol-grown R.rhodochrous PNKb1

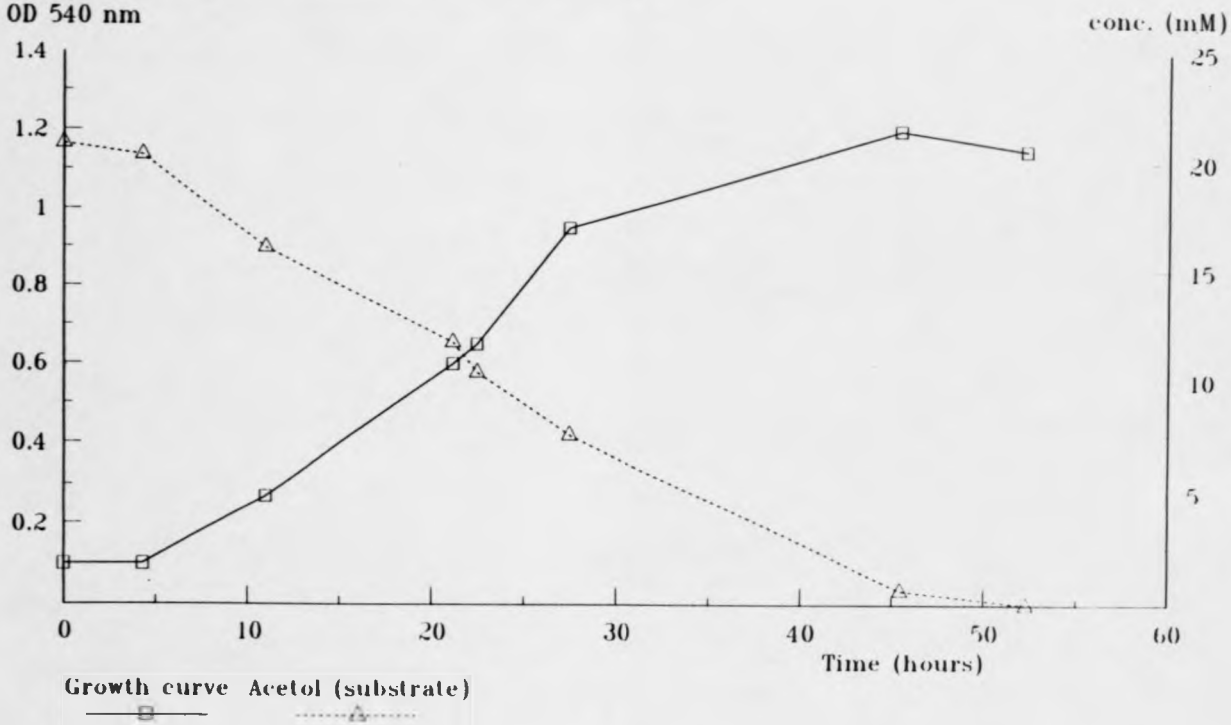
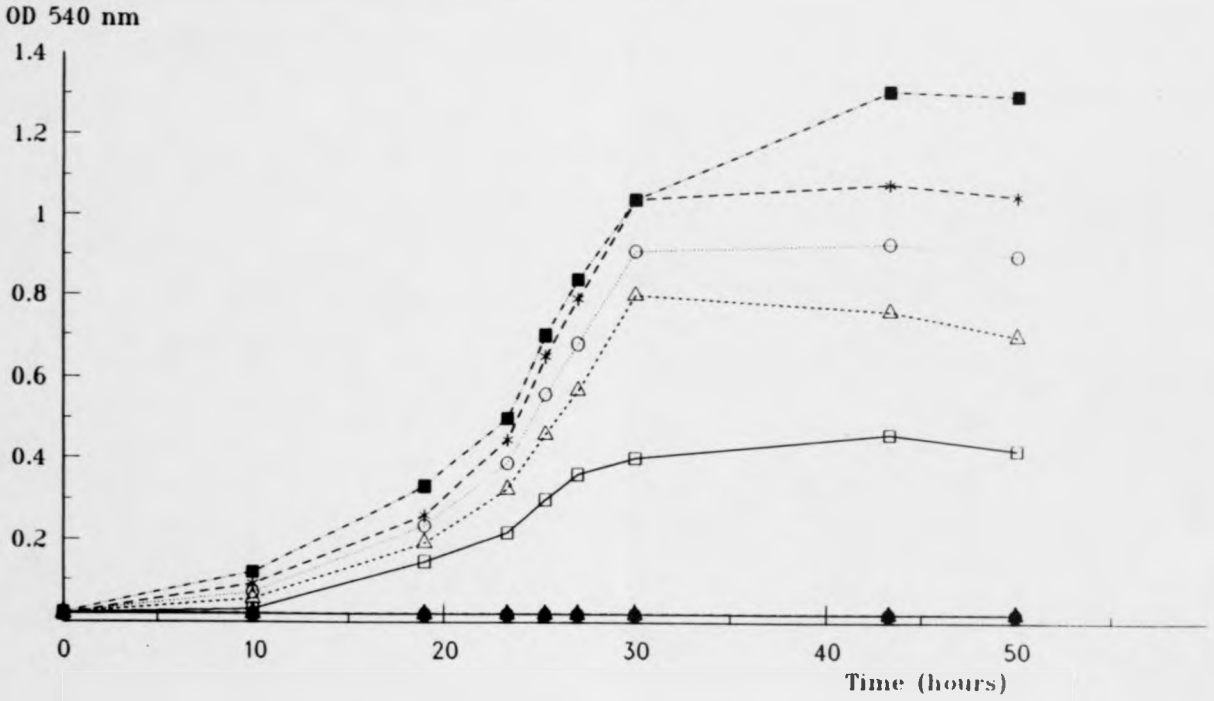


Figure 3.7 Ability of R.rhodochrous PNKb1 to grow on methyl acetate



Growth curve using various concentrations of methyl acetate;

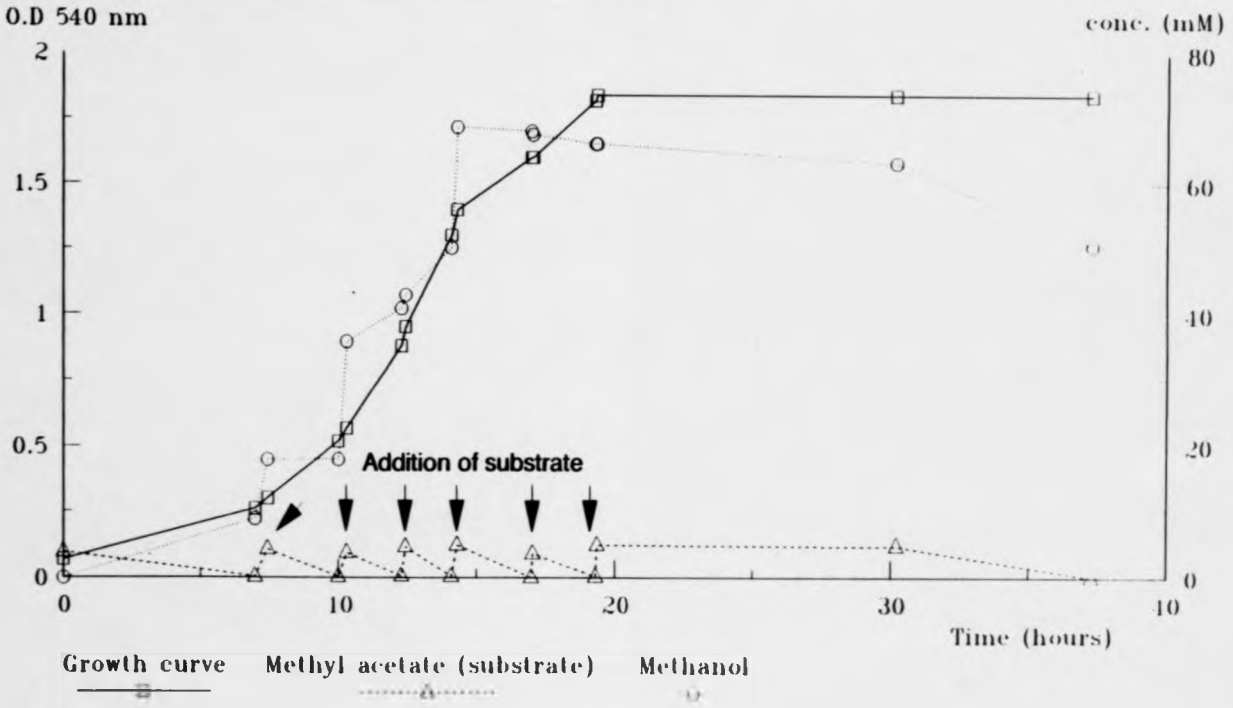
0.01 % (v/v) 0.02 % (v/v) 0.025 % (v/v) 0.03 % (v/v) 0.04 % (v/v) 0.05 % (v/v) 0.1 % (v/v)

was however observed at 0.1 or 0.05% (v/v), while slightly slower growth was noticed at 0.01% (v/v). R.rhodochrous PNKb1 was grown on methyl acetate and the culture supernatant was analyzed. Although methanol was detected in the supernatant of methyl acetate-grown cells (Fig. 3.8), it did not accumulate in culture supernatants of propane, propan-2-ol or acetone-grown cells. These results indicate that methyl acetate has a different and separate pathway and is not an intermediate in the metabolism of propane. Protein profiles of cell-free extracts of methyl acetate-grown cells were shown to be different from that obtained from propane-grown cells (section 3.2). The fate of methanol in the metabolism of methyl acetate-grown cells was not investigated. However, PNKb1 neither grows on nor oxidizes methanol under all growth conditions tested.

3.7 Summary

Studies have shown that similar polypeptides which may be components of a common oxygenase were synthesized by R.rhodochrous PNKb1 after growth on propane, acetol and acetone. Propan-2-ol and 1,2-propanediol-grown cells also synthesized polypeptides that were found in propane-grown cells. This could demonstrate a relationship between the metabolism of the above compounds. However, cell-free extracts of methyl acetate-grown cells show different patterns from that observed with propane-grown cell extracts indicating that methyl acetate is metabolised by a separate pathway and is not involved in the metabolism of propane.

Figure 3.8 Excretion of methanol from methyl acetate-grown R.rhodochrous PNKb1



NAD^+-dependent secondary alcohol dehydrogenase was synthesized after growth on propane, intermediates of the subterminal (including acetone) and 1,2-propanediol. Only low amounts of this enzyme were observed in acetone- and 1,2-propanediol-grown cells when compared with propane-grown cells.

Elevated levels of NAD^+-dependent secondary alcohol dehydrogenase were only observed in propane and propan-2-ol-grown cells whereas only very low activities of diol dehydrogenase were found. This suggests the involvement of more than one alcohol dehydrogenase in the metabolism of propane by R.rhodochrous PNKb1.

Propan-2-ol accumulated in the supernatant of propane-grown cells in the presence of ammonium chloride or phosphate. Nevertheless, no other alcohols or potential intermediates were detected. This indicates that the subterminal oxidation of propane is a major route in the metabolism of propane by R.rhodochrous PNKb1.

Analysis of supernatants from cultures grown on potential propane oxidation intermediates demonstrates that propan-1-ol, propan-2-ol and 1,2-PDL are involve in the metabolism of propane (see Fig. 3.9). A transient accumulation of acetol was detected from the supernatant of 1,2-PDL-grown cells. The excreted acetol was consumed only when the medium was lacking the substrate 1,2-PDL, suggesting that acetol could be an intermediate of 1,2-PDL metabolism. Therefore, ther is

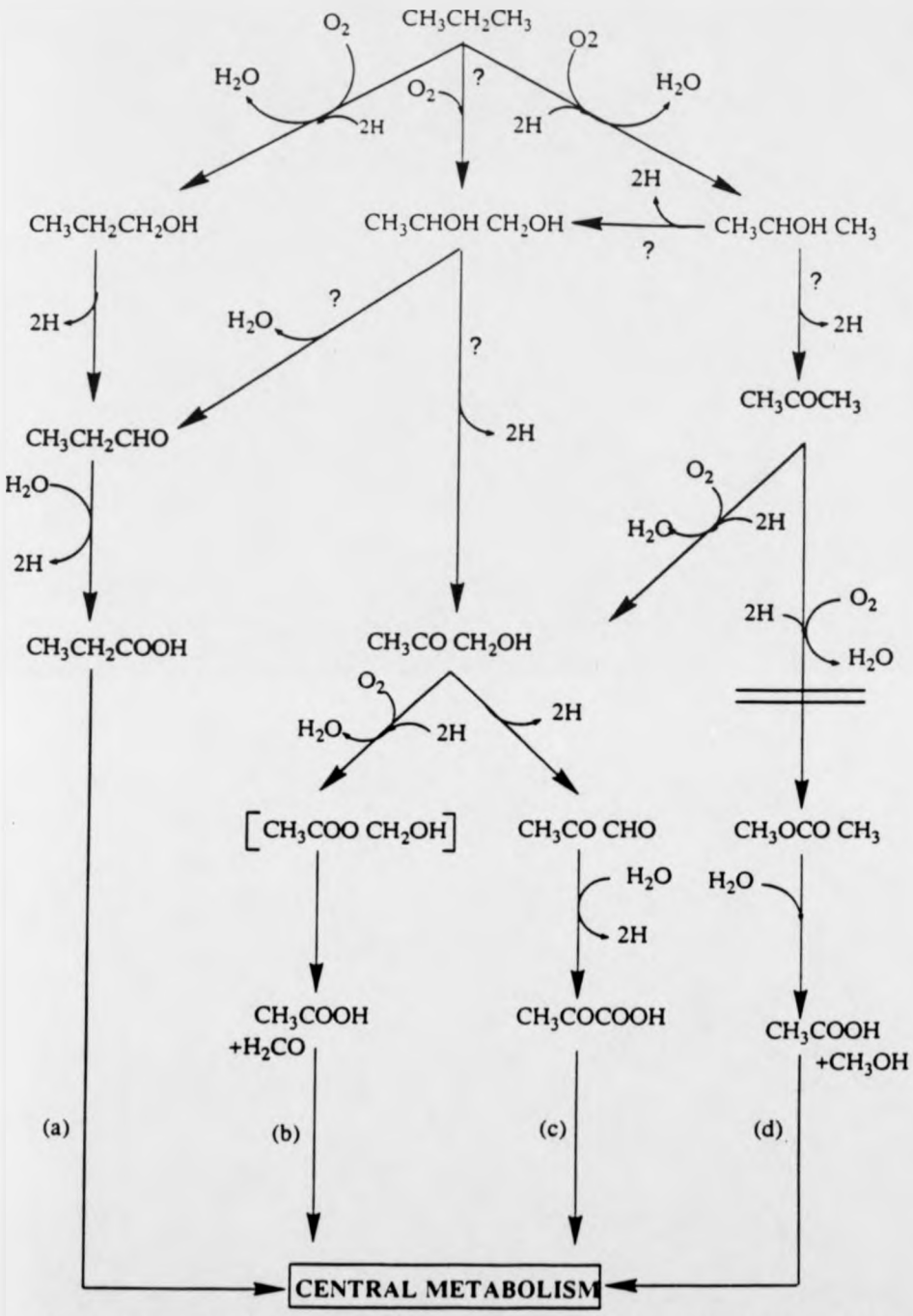


Figure 3-9 Proposed propane oxidation pathway by Rhodococcus rhodochrous PNKb1. (a) Terminal oxidation via propanoate; (b) sub-terminal oxidation via acetol and hydroxymethyl acetate; (c) sub-terminal oxidation via pyruvate; (d) sub-terminal oxidation via methyl acetate.

a possibility that the first step of propane oxidation is catalysed by a novel dioxygenase. This hypothesis could still be confirmed if the propane mono(di)-oxygenase was purified. However, the oxygenase activity was extremely labile even in the presence of numerous stabilization agents (Woods, 1988).

Acetone only transiently accumulated in the supernatant of propan-2-ol-grown cells and was further metabolized only when the medium was lacking in the substrate (propan-2-ol).

Unfortunately, no potential intermediates were detected from the supernatant of cultures grown on propane, acetone or acetol.

A very high concentration of methanol accumulated in the supernatant of methyl acetate-grown cells while methanol was not excreted from cultures grown on propane, propan-2-ol or acetone. Also methanol was not oxidized or metabolized (as growth substrate) by PNKb1. This indicates clearly that methyl acetate is metabolized by a separate pathway and is not an intermediate in the propane oxidation pathway (Fig. 3.9).

3.8 Growth on propane analogues

3.8.1 Introduction

The ability of R.rhodochrous PNKb1 to grow on compounds similar in structure to propane was tested. 1-Chloropropane, 2-chloropropane and 1,2 di-chloropropane are very similar to the propane molecule except for the addition of chlorine at the first and/or the second carbon atom (Fig. 3.10). If the above compounds are metabolized by R.rhodochrous PNKb1, one of the following might occur:

a) 1-Chloropropane could be degraded into 1-chloro-2-propanol or propan-1-ol in an oxidation or a dechlorination (Fig. 3.10). Propan-1-ol could be subsequently metabolized to propanal and then to propanoate, see Fig. 3.9.

b) 2-Chloropropane could be oxidized into 2-chloro-1-propanol or propan-2-ol (Fig.3.10) which in turn could be oxidized to acetone or acetol, see Fig. 3.9.

c) 1,2 di-chloropropane could be oxidized into 1-chloro-2-propanol or 2-chloro-1-propanol, see Fig.3.10.

The above pathways might occur if a propane monooxygenase (PMO) is involved in R.rhodochrous PNKb1. However, 1,2-PDL could also be produced if R.rhodochrous PNKb1 possesses a propane dioxygenase (Fig.3.10).

3.8.2 Results and discussion

The ability of R.rhodochrous PNKb1 to grow on 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane

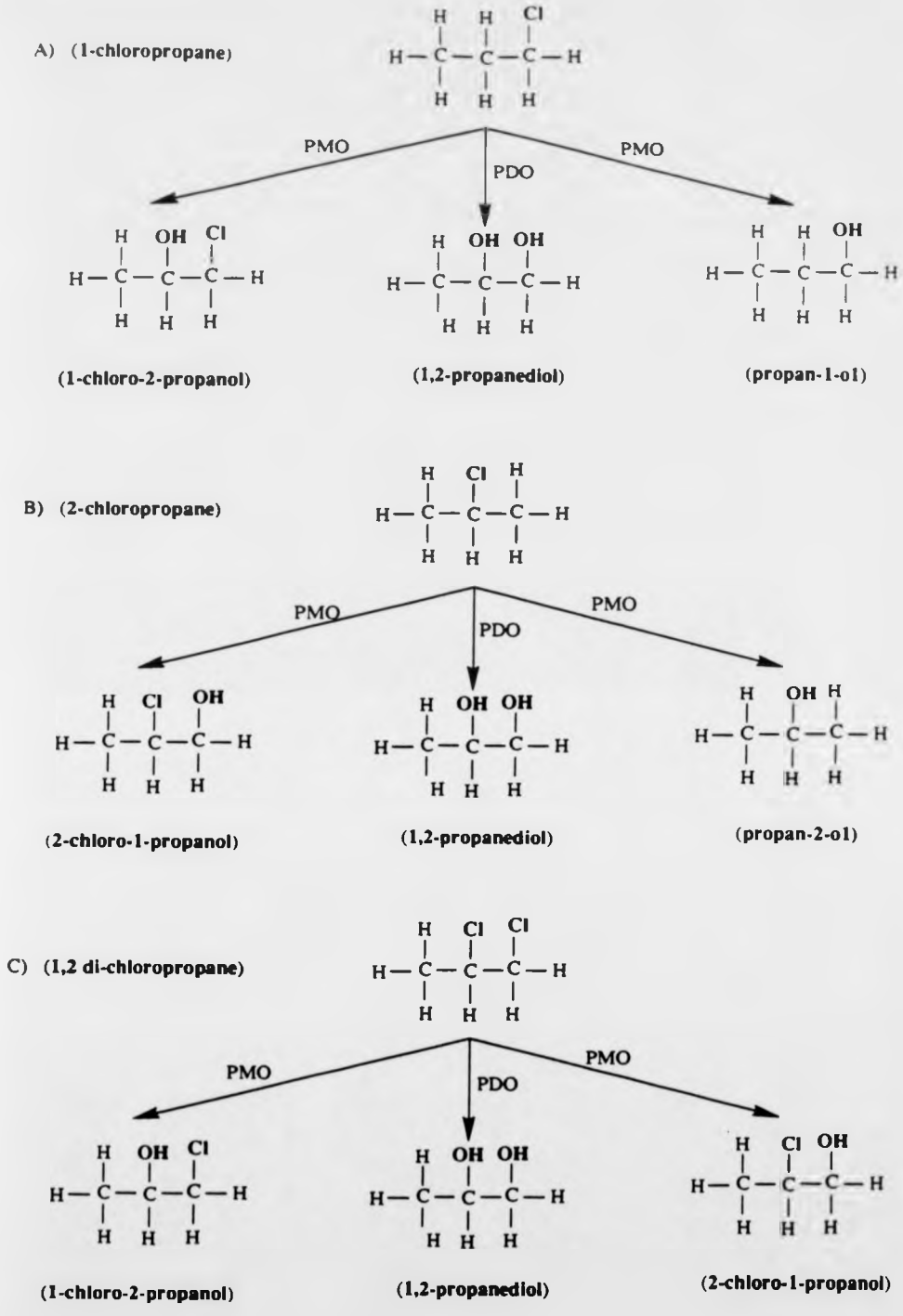


Figure 3-10 Proposed pathways for the degradation of chloropropanes by R.rhodochrous PNKb1.

is shown in Table 3.4. No growth was observed when the above compounds were used as the only source of carbon and energy [even below 0.025% (v/v)]. Similarly, no growth was observed when cells were grown on 1-chloropropane, 2-chloropropane or 1,2 di-chloropropane in the presence of propane. Good growth was noted when R.rhodochrous PNKb1 was grown on 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane in the presence of 0.1% succinate. These results indicate that 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane inhibit the oxidation of propane but not growth on other substrates such as succinate. Unfortunately it had been hoped that only one or possibly two of the substituted propane molecules would inhibit propane oxidation, thus giving a differential effect e.g. if 2-chloropropane and not 1-chloropropane inhibited then this might suggest that a chlorine atom at the subterminal carbon atom was blocking the active site of the enzyme.

R.rhodochrous PNKb1 was also grown on 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane in the presence of propane and 0.035% succinate (w/v) as growth supporting substrate. Growth under these condition, obtained a maximum OD_{540} of approximately 0.4, might affect the synthesis of propane-specific polypeptides.

Figure 3.11 shows the SDS-PAGE of soluble protein from cells grown on low concentrations of growth supporting substrate [0.035% (w/v) succinate] with 1-chloropropane, 2-chloropropane or 1,2 di-chloropropane and in the absence (-)

Table 3.4 Ability of R.rhodochrous PNKb1 to grow on 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane

Growth substrate	Growth
1-Chloropropane	-
2-Chloropropane	-
1,2 di-Chloropropane	-
1-Chloropropane + Propane	-
2-Chloropropane + Propane	-
1,2 di-Chloropropane + Propane	-
1-Chloropropane + 0.1% Succinate*	+++
2-Chloropropane + 0.1% Succinate*	+++
1,2 di-Chloropropane + 0.1% Succinate*	+++
1-Chloropropane + Propane + 0.035% Succinate*	+
2-Chloropropane + Propane + 0.035% Succinate*	+
1,2 di-Chloropropane + Propane + 0.035% Succinate*	+

- No growth + Weak growth +++ Good growth

* (w/v)

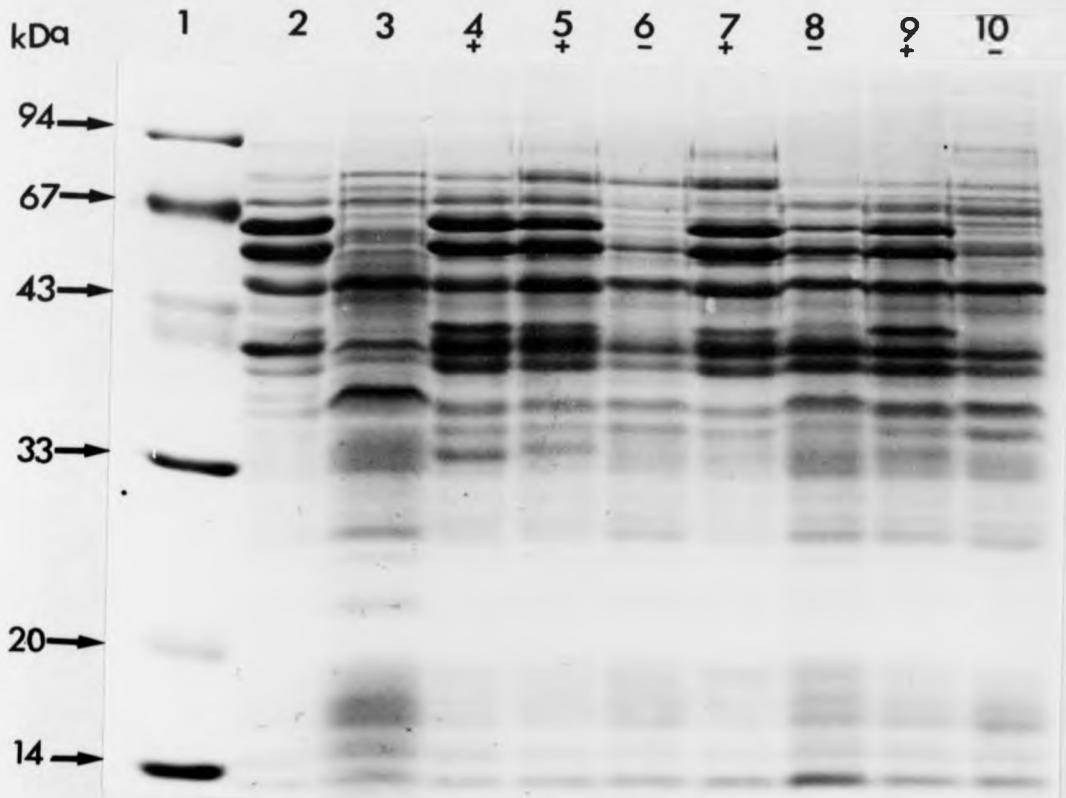
Figure 3.11 SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on a low concentration of succinate in the absence (-) and presence (+) of propane with 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane.

Track	Growth substrate
1	(Molecular weight markers)
2	Propane
3	Succinate**
4	Propane + succinate*
5	Propane + succinate* + 1-chloropropane
6	Succinate** + 1-chloropropane
7	Propane + succinate* + 2-chloropropane
8	Succinate** + 2-chloropropane
9	Propane + succinate* +1,2 di-chloropropane
10	Succinate** + 1,2 di-chloropropane

* 0.035% (w/v)

** 0.1% (w/v)

100 μg protein in each track


kDa
1
2
3
4 +
5 +
6 −
7 +
8 −
9 +
10 −
94
67
43
33
20
14

and presence (+) of propane. Results obtained from cells grown on low concentration of succinate in the presence (+) of propane in addition to 1-chloropropane or 1,2 di-chloropropane shows the synthesis of propane-specific polypeptides. However, a specific 69 kDa polypeptide was not synthesized when cells were grown on 0.035% succinate (w/v) in the presence of propane, and 2-chloropropane. Ashraf, 1990 reported that 0.1% succinate (w/v) represses the induction of propane specific polypeptides. Propane-specific polypeptides were synthesized when cells were grown on 0.1% succinate (w/v) with 2-chloropropane and in the absence of propane(-). However, none of the specific polypeptides were synthesized when cells were grown on 0.1% succinate (w/v) in addition to 1-chloropropane or 1,2 di-chloropropane and in the absence of propane(-). Results from Fig. 3.12 do not give clear evidence for the type of propane oxygenase system unless PNKb1 had grown on the above chloropropane compounds.

The ability of propane-grown PNKb1 to oxidize 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane was tested using oxygen electrode technique. All three chloropropane compounds were not oxidized by whole cells of propane-grown PNKb1 (Table 3.5). The effect of these compounds on the oxidation of propane by whole cells after growth on propane is shown in Table 3.6. 1-Chloropropane exhibits a marked effect on the oxidation of propane. However, a lack of inhibition by 2-chloropropane and relatively with 1,2 di-chloropropane was observed.

Table 3.5 Ability of R.rhodochrous PNKb1 to oxidize propane analogues after growth on propane

Assay substrate	nmol O_2 consumed min^{-1} mg dry weight cells^{-1}
Propane	19
1-Chloropropane	0
2-Chloropropane	0
1,2 di-Chloropropane	0

Table 3.6 Effect of propane analogues on O_2 consumption by whole cells of propane-grown R.rhodochrous PNKb1

Inhibitor	Conc. in assay (mM)	Relative activity (%)
None	-	100
1-Chloropropane	0.49	44
2-Chloropropane	0.73	85
1,2 di-Chloropropane	0.41	69

100% activity is 19 nmol oxygen consumed min^{-1} mg dry weight cells^{-1}

3.8.3 Summary

R.rhodochrous PNKb1 failed to utilize or oxidize propane analogues such as 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane. These compounds were shown to be specifically inhibiting propane oxidation. Growth was only observed when cells were grown on succinate [(0.035% (w/v)] in the presence of chloropropane compounds, while no growth was noted when succinate was removed. SDS-PAGE of soluble proteins of R.rhodochrous PNKb1 after growth on propane analogues compounds under propane-inducing conditions shows the synthesis of propane-specific polypeptides. Nevertheless, one of the propane-specific polypeptides of approximately 69 kDa was not synthesized when cells were grown on the above conditions in the presence of 2-chloropropane. The propane-specific polypeptides were synthesized when cells were grown on 0.1% (w/v) succinate with 2-chloropropane and in the absence of propane . Ashraf (1990) demonstrated that growth on 0.1% (w/v) succinate in the presence or the absence of propane did not synthesize the propane-specific polypeptides. This indicates that succinate represses the induction of the propane oxygenase. The reason for the induction of the propane-specific polypeptides under the above condition is not known.

CHAPTER 4

ENZYMOLOGY OF R.RHODOCHROUS PNKB1

4.1 Introduction

The ability of R.rhodochrous PNKb1 to oxidize propene to 1,2-epoxypropane was used as an indicator for propane oxygenase activity. It has been postulated that epoxide formation is catalysed by the propane oxygenase (Woods and Murrell, 1989).

In this study, K_m and V_{max} of propene oxidation were determined in the presence and the absence of propane, using propane-grown cells. Propane was shown to be a competitive inhibitor of propene oxidation, presumably binding at the same active site on the propane oxygenase.

Epoxide formation from propene was only detected using propane and acetol-grown cells, demonstrating that a common oxygenase might be responsible for the oxidation of propane and acetol. To prove this suggestion, the effect of various potential inhibitors on the formation of 1,2-epoxypropane from propene by whole cells of propane and acetol-grown R.rhodochrous PNKb1 was investigated. This was followed by partial purification of acetol monooxygenase from acetol-grown cells.

4.2 Formation of 1,2-epoxypropane from propene

Figure 4.1 and 4.2 show the ability of PNKb1 to epoxidate propene to 1,2-epoxypropane in cell-free extracts and by whole cell suspensions of propane and acetol-grown cells

Figure 4.1 Formation of 1,2-epoxypropane from propene in cell-free extracts of propane and acetol-grown R.rhodochrous PNKb1

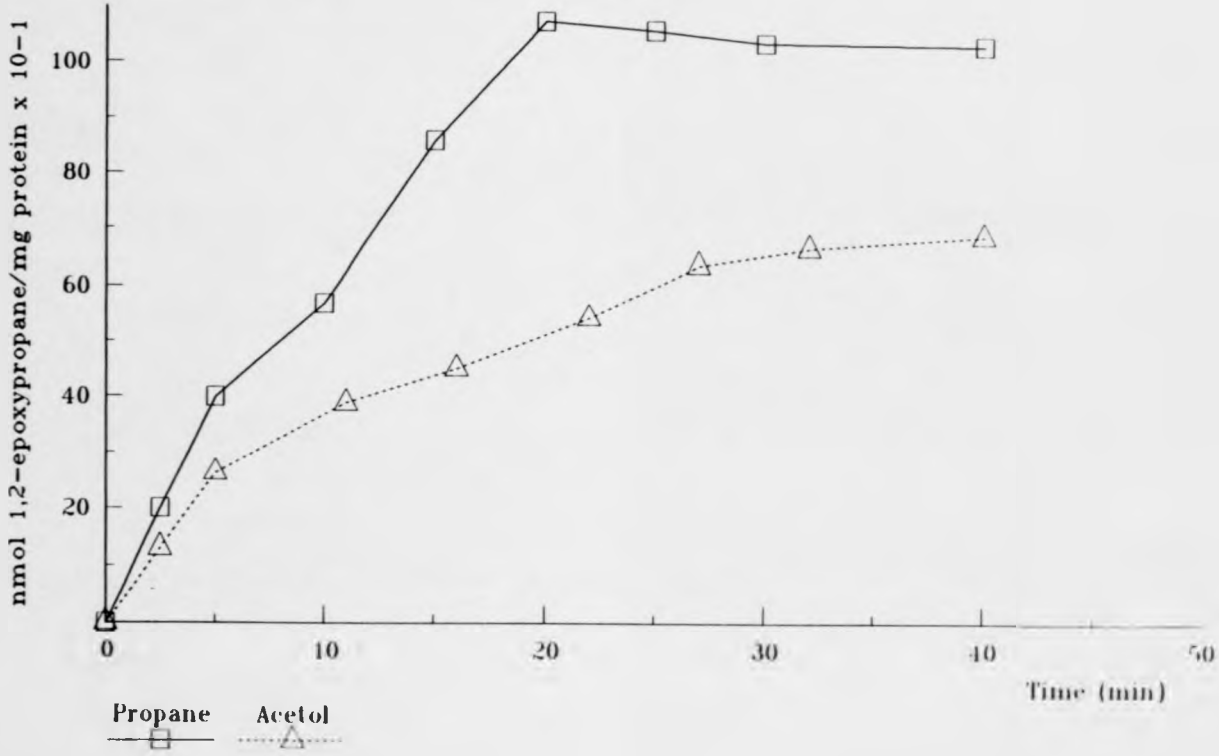
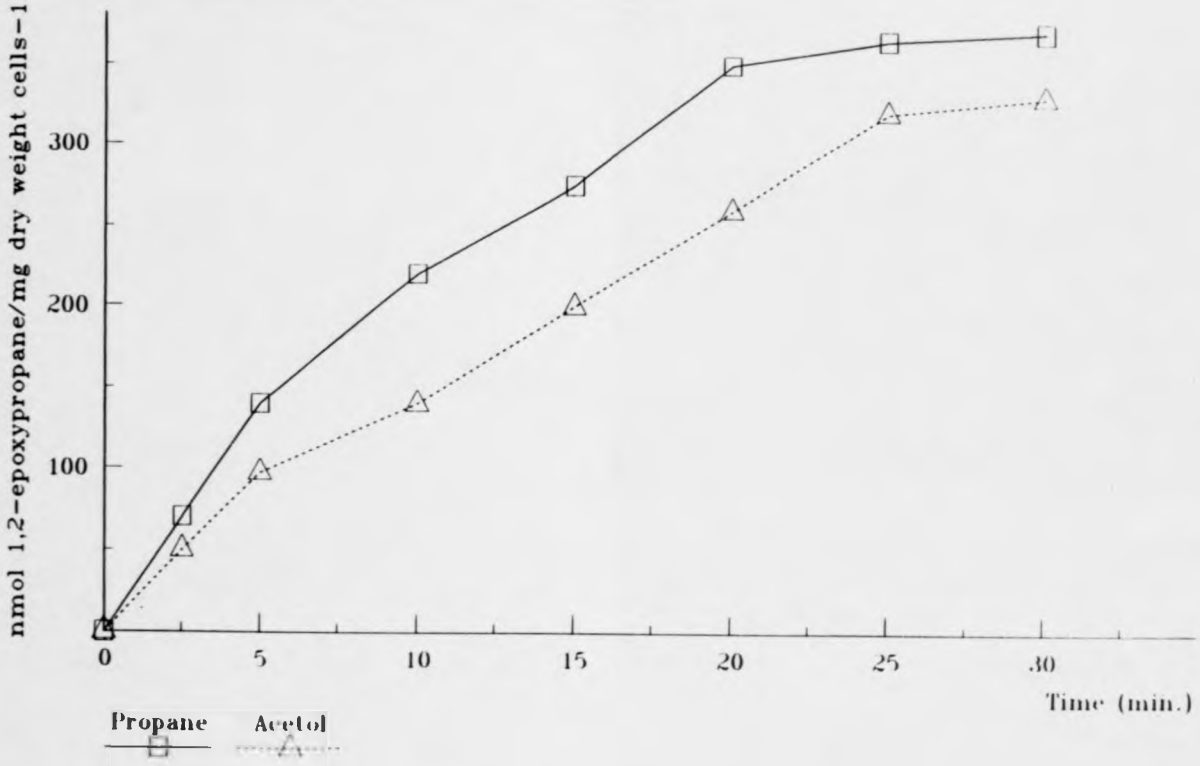


Figure 4.2 Formation of 1,2-epoxypropane from propene by resting cell suspensions of propane and acetol-grown R.rhodochrous PNKb1



respectively. Table 4.1 shows the rate of 1,2-epoxypropane formation in cell-free extracts of R.rhodochrous PNKb1 after growth on propane and various oxidation intermediates. The rates obtained with cell-free extracts of propane-grown cells were slightly higher than those obtained from extracts of acetol-grown cells. This might imply that propane and acetol are oxidized by the same or similar oxygenase system. However, 1,2-PDL and acetone-grown cells did not form 1,2-epoxypropane in cell-free extracts (Table 4.1). The lack of epoxidation activity in 1,2-PDL-grown cells might suggest that propane oxygenase is not active after growth on 1,2-PDL. Alternatively an oxygenase which cannot perform epoxidation reactions might be present. Cerniglia *et al.* (1976) reported that some strains of propane-utilizers formed acrylic acid from propene rather than 1,2-epoxypropane. The lack of activity with acetone-grown cells may indicate that acetone-grown cells possess a different oxygenase system. Alternatively acetone oxygenase might oxidize propene to allyl alcohol or acrylic acid rather than 1,2-epoxypropane.

4.3 K_m and V_{max} determination of propene oxidation by propane-grown cells

Woods (1988) reported that addition of propane gas to propane-grown R.rhodochrous PNKb1 inhibited the formation of 1,2-epoxypropane from propene, indicating that propane and propene are competing for the same active site on propane oxygenase. However, this conclusion cannot be drown from

Table 4.1 Formation of 1,2-epoxypropane from propane in cell-free extracts of R.rhodochrous PNKb1 after growth on propane and potential oxidation intermediates.

Growth substrate	Formation of 1,2-epoxypropane from propene[a]
Propane	80
Acetol	53
Acetone	0
1,2-propanediol	0

[a]Rates quoted as nmol 1,2-epoxypropane formed min^{-1} mg protein $cells^{-1}$

this type of assay. Figure 4.3 shows the effect of adding propane during the conversion of propene to 1,2-epoxypropane by resting cell suspensions. Addition of 1.5 ml propane gas gave 50% inhibition of the formation of 1,2-epoxypropane from propene. V_{max} and K_m for propene oxidation were determined in the absence of inhibitor (propane). Thereafter, a fixed amount of propane gas (1.5 ml) was added whilst assaying the conversion of propene to 1,2-epoxypropane using various concentrations of the substrate (propene) and the activity was measured (Fig. 4.4). V_{max} of propene epoxidation in the absence and the presence of propane as inhibitor were 21.7 and 21.98 nmol min^{-1} mg dry weight $cells^{-1}$ respectively. However, the K_m value for propene in the absence and the presence of propane as inhibitor was altered (0.08 and 0.18 mM, respectively). This clearly shows that propane and propene are competing for the same active site on propane oxygenase.

4.4 Inhibition of propane/acetol oxygenase activity

The effect of various inhibitors on epoxide formation by whole cells and cell-free extracts of propane-grown R.rhodochrous PNKb1 was used as a measure of propane oxygenase activity (Woods, 1988). Previous results contained in this thesis suggests that propane and acetol might be oxidized by the same or similar oxygenase system. Therefore, the effect of potential inhibitors on epoxide formation by whole cells of propane and acetol-grown cells was measured (Table 4.2). Inhibitor profiles of propane-grown cells were

Figure 4.3 Effect of adding propane during the formation of 1,2-epoxypropane from propene by whole cells of propane-grown R.rhodochrous PNKb1

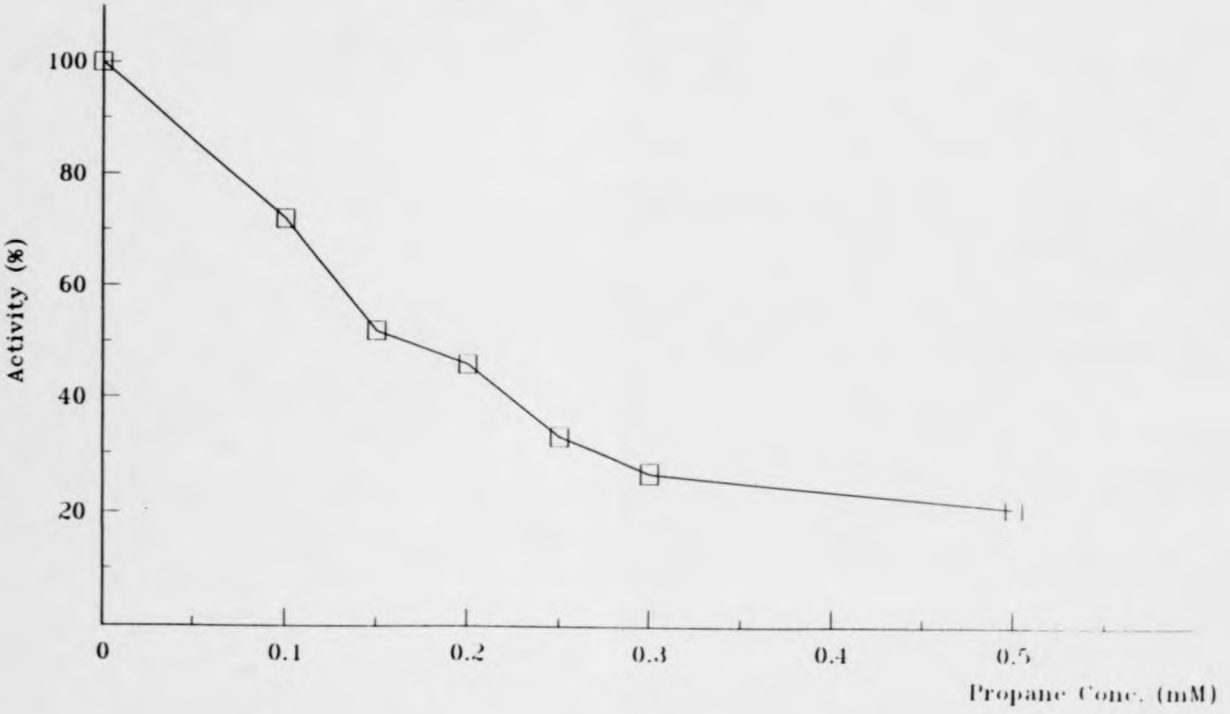


Figure 4.4 Lineweaver–Burk plot showing the effect of propane as inhibitor on the epoxidation of propene by whole cells of propane-grown PNKb1

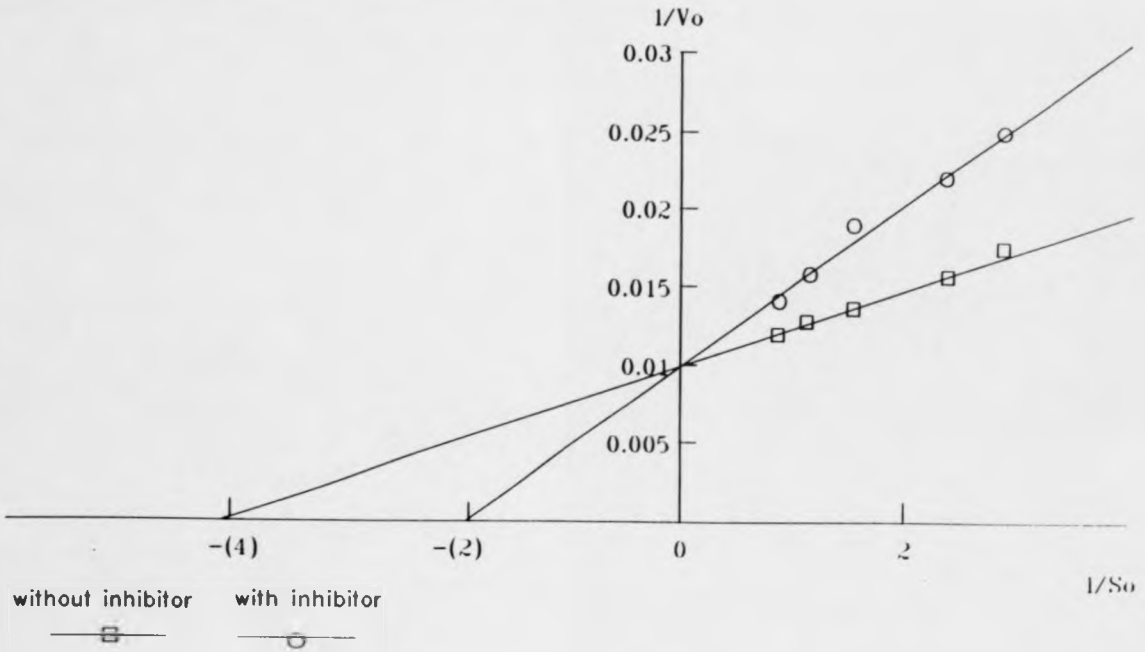


Table 4.2 Effect of potential inhibitors on the formation of 1,2-epoxypropane from propene by resting cell suspensions of propane and acetol-grown R.rhodochrous PNKb1

Inhibitor	Concentration (mM)	Relative activity (%)	
		Growth substrate	
		Propane	Acetol
None	-	100	100
Azide	1	90	86
KCN	5	52	52
	10	40	41
Na_2EDTA	10	64	69
8-Hydroxyquinoline	10	93	54
2-Mercaptoethanol	10	80	66
Carbon monoxide	0.5	100	100
Acetylene	7.5	100	100

100% activity of propane and acetol are 14 and 10 nmol 1,2-epoxypropane formed min^{-1} mg dry weight $cells^{-1}$, respectively

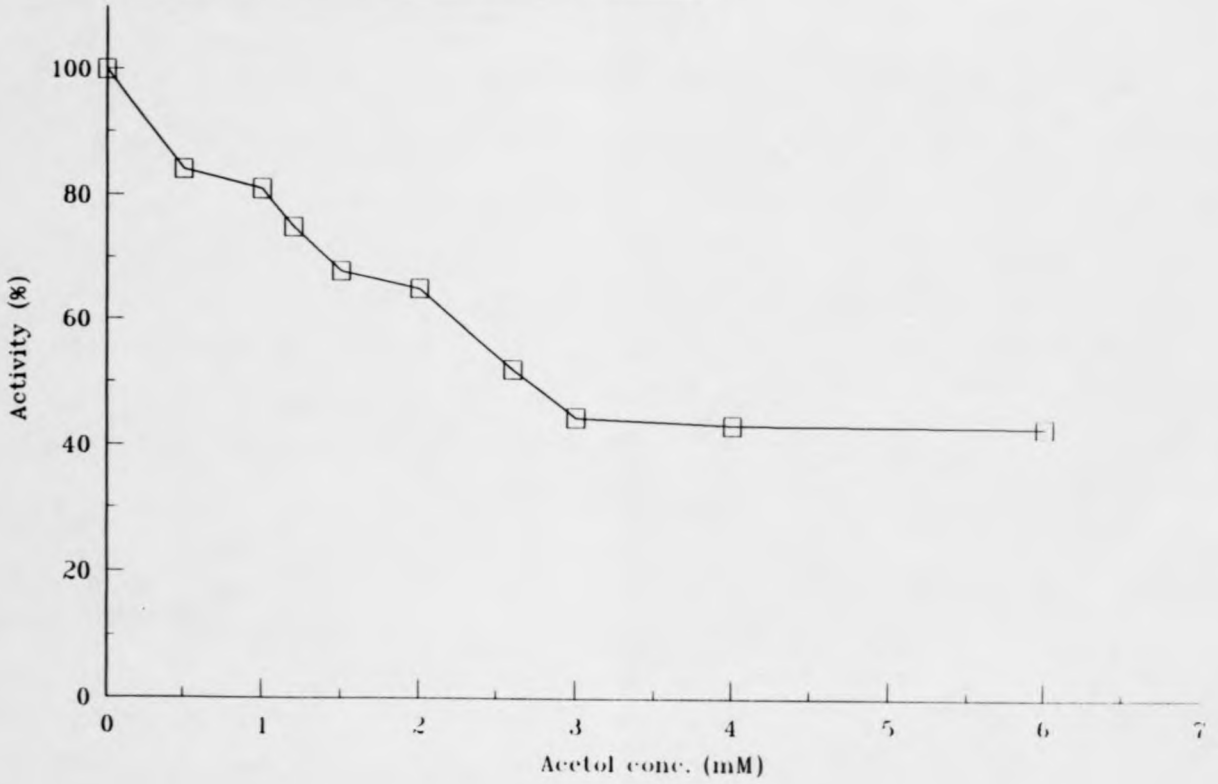
comparable with those given by Woods (1988). Propene oxidation by propane and acetol-grown cells was affected to a similar extent by the same inhibitors. However, propene oxidation by acetol-grown cells were inhibited to a great extent with 8-hydroxyquinoline and 2-mercaptoehanol when compared with propane-grown cells. Propane oxygenase activity was totally unaffected by the presence of carbon monoxide and acetylene but was susceptible to inhibition by cyanide. Whereas the activity was to a large extent unaffected by azide, 8-hydroxyquinoline and 2-mercaptoethanol, it was however affected by Na_2EDTA. Lack of inhibition by carbon monoxide indicates that R.rhodochrous PNKb1 does not possess a cytochrome P_{450}-type oxygenase system.

4.5 Partial purification of acetol monooxygenase

4.5.1 Introduction

Previous results in section 4.2 showed that propane and acetol-grown cells oxidize propene to 1,2-epoxypropane at a relatively similar rate. In addition, inhibitor studies showed that propane and acetol-grown cells were affected by the same inhibitors (section 4.4). Also, formation of 1,2-epoxypropane from propene in cell-free extracts of propane-grown cells was inhibited by the addition of acetol (see Fig. 4.5). These results suggest that an oxygenase system might be responsible for the oxidation of both propane and acetol. Previous attempts to purify propane oxygenase from

Figure 4.5 Effect of adding acetol during the formation of 1,2-epoxypropane from propene in cell-free extracts of propane-grown PNKb1



propane-grown cells were unsuccessful due to the unstable nature of this enzyme ($T_{1/2}$ at 4°C = 150 minutes) (Woods, 1988). However, acetol oxygenase activity from acetol-grown cells is very stable in ice (acetol oxygenase activity was only decreased 5% after 18 hours incubation at 0°C). This enzyme was also fairly stable at -70°C for several weeks. Due to the great stability of acetol oxygenase activity from acetol-grown cells, attempts were made to purify this enzyme using various techniques.

4.5.2 Results and discussion

NADH-dependent acetol monooxygenase activity was detected in soluble cell-free extracts of acetol-grown R.rhodochrous PNKb1 using a Beckman DU-70 spectrophotometer. Neither NAD^+ nor $NADP^+$-dependent acetol dehydrogenase activity could be detected in cell-fee extracts of acetol-grown cells. Similar results were reported with propane-grown cells, indicating that acetol is not metabolized to pyruvate (Woods and Murrell, 1989) (Fig. 1.8). These observations suggest that acetol could be oxidized by an acetol monooxygenase (AMO) to an unstable intermediate, hydroxymethylacetate, which is subsequently metabolized to acetate and formaldehyde (Fig. 1.8). Hartmans and de Bont (1986) described a novel NADPH-dependent acetol monooxygenase from Mycobacterium Py1 which cleaves acetol into acetate and formaldehyde (see section 1.3.3.6 and 1.4.1.4).

The optimum pH of "acetol monooxygenase" in cell-free extracts of acetol-grown cells was 7.0. The buffer used was 20 mM Tris-HCl. However, 20 mM phosphate buffer solution, pH 7.0 gave 31% of the activity observed with Tris-HCl buffer. So, 20 mM Tris-HCl was used as a buffer solution with all further experiments. Acetol monooxygenase had an optimum temperature of 40°C for activity.

Attempts to purify acetol monooxygenase activity from acetol-grown PNKb1 were made using ion-exchange chromatography and gel filtration techniques. This resulted a partial purification of acetol monooxygenase. Figure 4.6 shows the elution of the enzyme from an ion-exchange column (Mono-Q) using an increasing sodium chloride gradient (0, 10, 20, 50, and 100%) which gave rise to three major protein peaks (as determined by absorbance of 280 nm). Woods (1988) also observed three major protein peaks from cell-free extracts of propane-grown PNKb1 using the ion-exchange chromatographic technique. Unfortunately, he could not detect the activity of propane oxygenase in those three major fractions. Recombining those major protein fractions did not result in detection of propane oxygenase activity. The reason for that failure was due to the instability of propane oxygenase at 4°C ($T_{1/2}$ = 150 mins). In this study AMO activity was observed with fractions 18 and 19, see Fig. 4.6. Fresh crude extracts were loaded to column DEAE-Sepharose. Three major protein peaks were also separated. AMO activity was measured in fractions 20-23 (Fig. 4.7). These fractions along with fractions 18 and 19 from the

Fig. 4.6 Elution profile of R.rhodochrous PNKb1 AMO from ion-exchange chromatography column (Mono-Q)

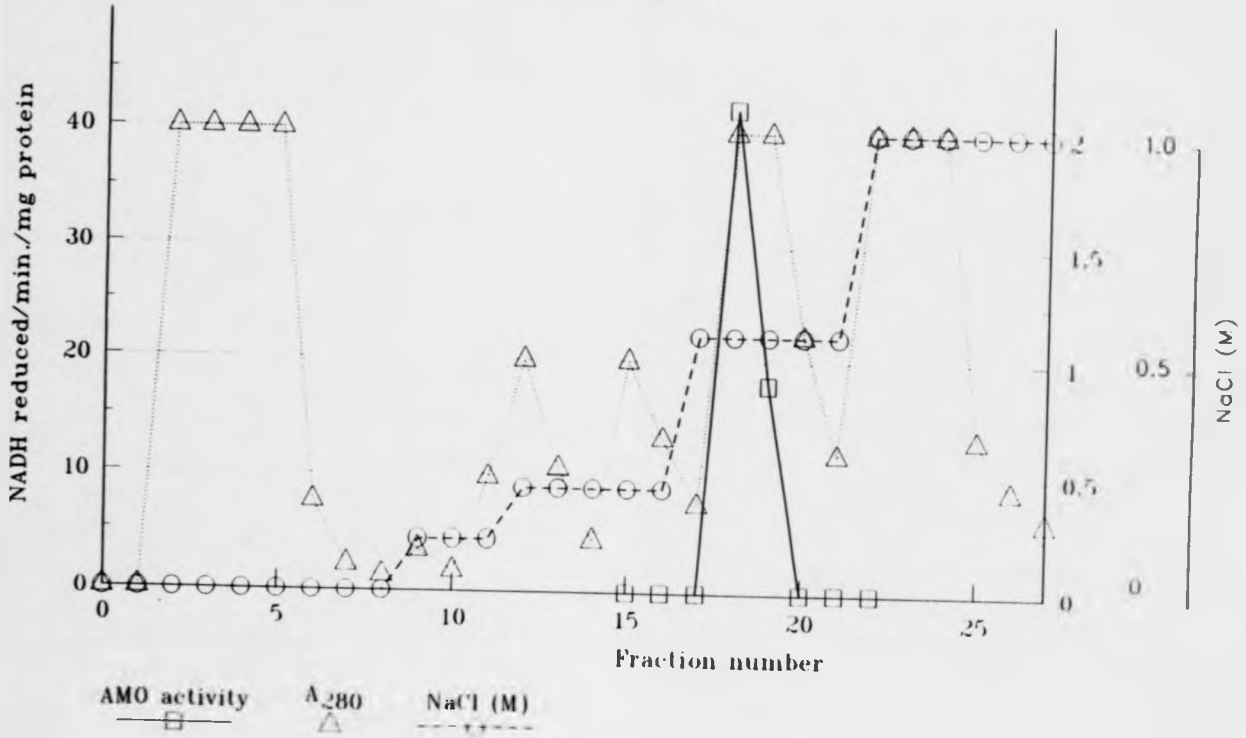
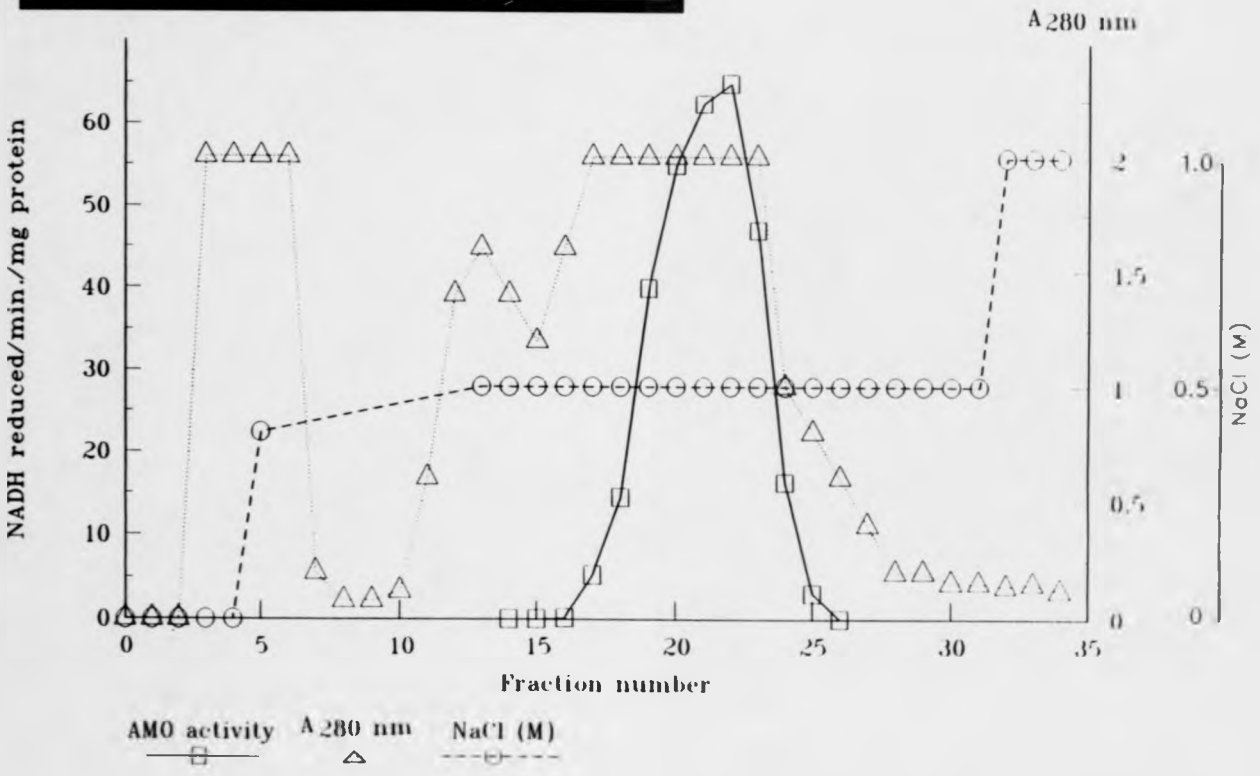


Fig. 4.7 Elution profile of R.rhodochrous PNKb1 AMO from ion-exchange chromatography column (DEAE-Sepharose)



Mono-Q column were concentrated and then applied to an S-200 gel filtration. This technique separates proteins on the basis of their size. The elution profile from the gel filtration column is shown in Fig. 4.8. Elevated AMO activity was detected in fractions 81-85. These fractions were concentrated and applied to a Mono-Q ion-exchange chromatography column. Three major protein peaks were observed (A, B and C) (Fig. 4.9). Unfortunately, AMO activity was not detected in any of the eluted major protein fractions. However, recombining all three protein fractions (A, B and C) gave 157 nmol NADH reduced min^{-1} mg protein $^{-1}$. AMO activity was also detected by the formation of 1,2-epoxypropane from propene. Combining protein fractions A and C gave similar results while half of that activity was measured when the protein fractions A and B were combined. This suggests that fractions of protein B could be contaminant of protein C. Surprisingly, SDS-PAGE of the eluted fractions of the three major protein from the ion-exchange column show no large differences in terms of protein synthesis profile to that obtained from crude extracts of acetol-grown cells. The purification of AMO activity must be investigated using further techniques.

4.6 Summary

<u>R.rhodochrous</u> PNKb1 shows the ability to epoxidate propene to 1,2-epoxypropane after growth on propane. Propane inhibited the formation of 1,2-epoxypropane from propene in resting cell suspensions of propane-grown cell. A similar

Fig. 4.8 Elution profile of samples from gel filtration (column S-200)

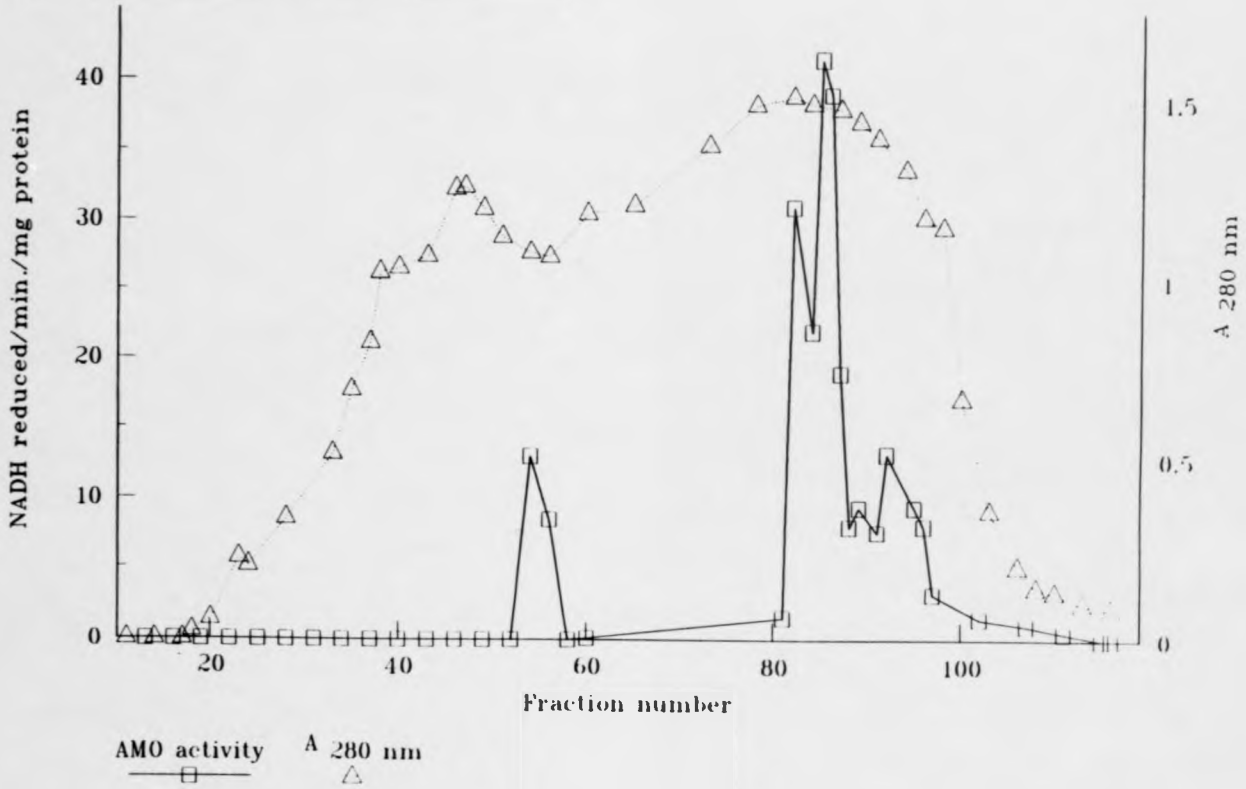
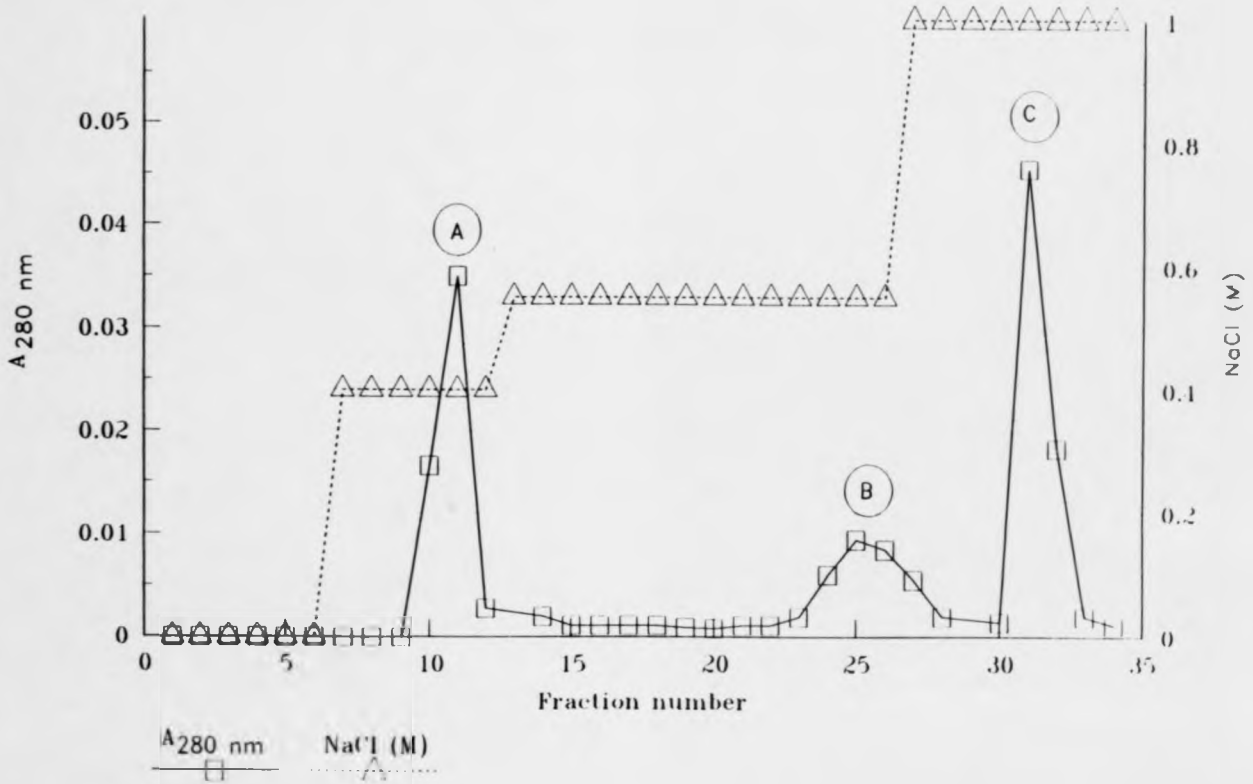


Fig. 4.9 Elution profile of R.rhodochrous PNKb1 AMO from ion-exchange chromatography (column Mono Q)



V_{max} of propene oxidation in the absence or the presence of inhibitor (propane) was determined while a different K_m was observed. This indicated that propane was a competitive inhibitor of propene oxidation acting at the same active site on propane oxygenase.

Cell-free extracts of propane and acetol-grown cells show the ability to form 1,2-epoxypropane from propene at a relatively similar rate. Further inhibition studies shows that propane and acetol-grown cells were relatively affected by the same inhibitors. This indicate a common oxygenase system for the oxidation of both propane and acetol.

The activity of AMO in cell-free extracts of acetol-grown PNKb1 found to be very stable at 0°C. The optimum temperature and pH of "acetol monooxygenase" were 40°C and 7, respectively. The use of gel filtration and ion-exchange chromatographic techniques resulted to partial purification of AMO. Three major protein fractions were separated from a Mono-Q column. None of those major protein fractions showed any AMO activity. However, combination studies between those protein fractions resulted a high AMO activity.

CHAPTER 5

ISOLATION AND CHARACTERIZATION OF MUTANTS DEFECTIVE IN PROPANE METABOLISM

5.1 Introduction

The purpose of this study was to account for the route (s) of propane oxidation in Rhodococcus rhodochrous PNKb1 using a genetic approach. Ashraf (1990) isolated several classes of NTG-generated mutants blocked at certain potential steps of the propane oxidation pathway, see Table 5.1. A brief synopsis of his characterization of NTG-generated mutants is summarized below:

alk$^-$ mutants were unable to grow on propane as a growth substrate, but retained the ability to utilize all potential intermediates of propane oxidation except acetol. This may indicate that a common oxygenase system is involved for both propane and acetol-grown cells.

Double mutants, alcAB$^-$ failed to utilize either propan-1-ol or propan-2-ol, but still grew on most of the other intermediates of propane oxidation, suggesting that propane produced a mixture of both propan-1-ol and propan-2-ol. Alternatively, a third route for propane oxidation might be involved e.g. via a 1,2-propanediol pathway.

Mutants which were defective in acetone metabolism (ket$^-$) failed to grow on acetone as growth substrate, but retained the ability to utilize propane and all potential intermediates of propane oxidation pathway. This suggests that acetone is not an intermediate of propane oxidation and

Table 5.1 Characterization of NTG-generated mutants

Class	No. of Mutants	Growth Substrates: Propane	Propan-1-ol	Propanal	Propanoate	1,2-Propanediol	Acetol	Propan-2-ol	Acetone
alk$^-$	8	-	+	+	+	+	-	+	+
alcA$^-$	4	-	-	+	+	+	+	+	+
alcB$^-$	4	-	+	+	+	+	+	-	+
alcAB$^-$	2	-	-	+	+	+	+	-	+
Ket$^-$	2	+	+	+	+	+	+	+	-

+ = Growth - = No growth

NB Wild-type grows on all intermediates

that propan-2-ol is metabolized to acetol and not acetone (Ashraf, 1990).

5.2 Mutagenesis

In order to obtain stable mutants, three chemical mutagenesis methods were used. R.rhodochrous PNKb1-Str^r_{20} was grown overnight in nutrient broth and then incubated with NTG, 1,2,7,8-diepoxyoctane (DEO) or 1,2,3,4-diepoxybutane (DEB). A survival rate of 50% was used to select for various classes of mutants blocked at certain steps in the metabolism of propane.

5.2.1 NTG-mutagenesis

Nutrient broth-grown cells of R.rhodochrous PNKb1-Str^r_{20} strain were incubated with NTG (150 $\mu g\ ml^{-1}$) for 12 minutes to give 50% kill (Ashraf, 1990). Under this condition, 1-2% auxotrophs were obtained using replica-plating techniques. Colourless or "white" colonies obtained were approximately 0.5%. Individual colonies after NTG-mutagenesis were replica-plated onto AMS plus succinate "master plates" and AMS plates which were incubated in an anaerobic jar with 50% (v/v) propane. Mutants which failed to grow on propane or its potential oxidation intermediates were tested by growth on liquid culture. Some mutants reverted after several subcultures in liquid medium. The reversion frequencies of NTG-generated mutants was 2.9×10^{-7}-10^{-9}. The phenotypic symbols used to describe the mutants isolated are: alk$^-$, no

growth on propane and acetol; *alcA*$^-$, no growth on propan-1-ol, *alcB*$^-$, no growth on propan-2-ol, *alcAB*$^-$, no growth on propan-1-ol and propan-2-ol; *alcC*$^-$, no growth on 1,2-propanediol; *Ket*$^-$, no growth on acetone; *aol*$^-$, no growth on acetol.

5.2.2 Penicillin enrichment

Penicillin enrichment is commonly used to increase the numbers of mutants after mutagenesis up to 100-fold. This method is based on the fact that penicillin kills only growing bacterial cells by interfering with the synthesis of the bacterial cell wall.

NTG-mutagenized cells of *R.rhodochrous* PNKb1-Str$^r_{20}$ or individual colonies of a specific mutant were incubated in AMS liquid medium supplemented with a particular intermediate (eg. 1,2-PDL, acetone ...etc.) along with penicillin-G (10,000 U ml^{-1}). Cultures were then left for 60-90 min. Control experiments showed that incubation of wild-type of *R.rhodochrous* PNKb1-Str$^r_{20}$ with penicillin-G stopped the growth.

Selection of NTG-generated mutants using penicillin enrichment did not significantly increase the number of mutants of *R.rhodochrous* PNKb1-Str$^r_{20}$ and so this method was not continued. The reasons for the lack of enrichment of mutants by this method are not known.

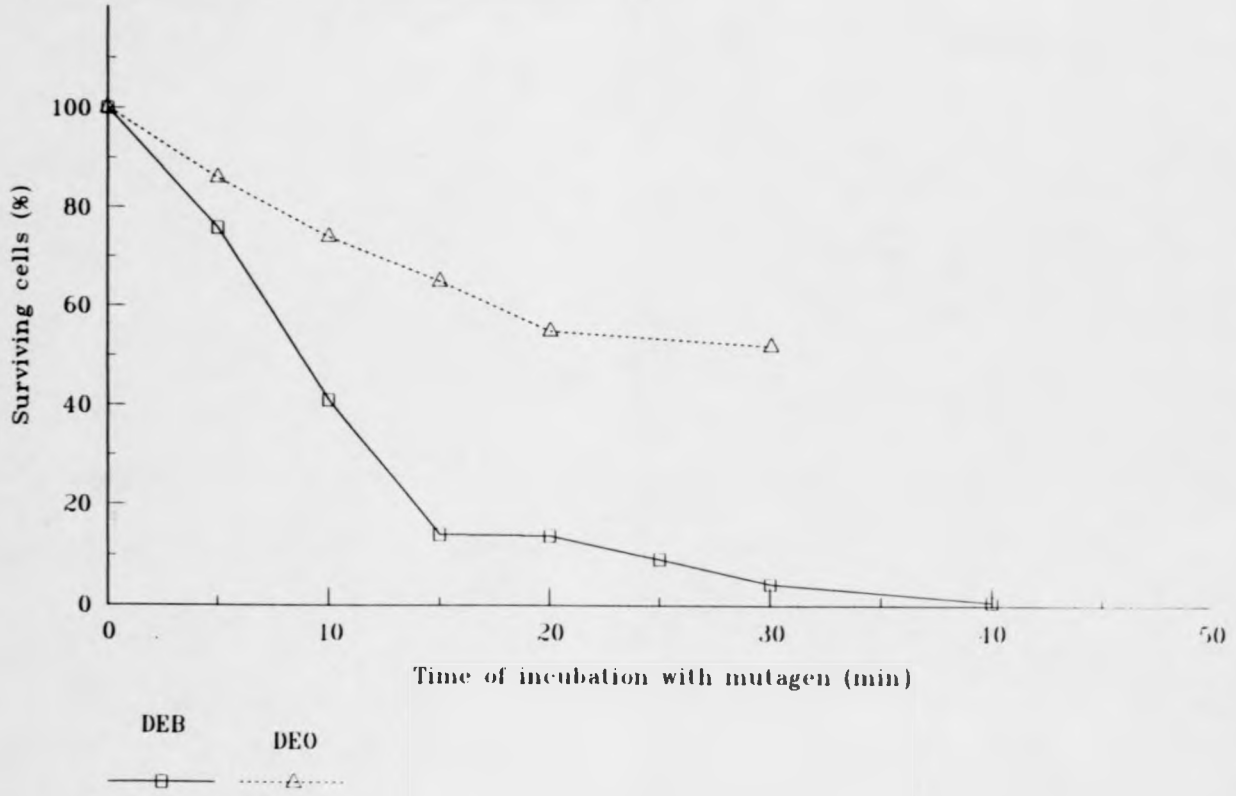
5.2.3 DEO & DEB-mutagenesis

Due to the relatively high reversion frequencies of mutants obtained by NTG-mutagenesis, 1,2,7,8-diepoxyoctane (DEO) or 1,2,3,4-diepoxybutane (DEB) were used in order to select for stable mutants. Very stable mutants were generated from Nocardia sp. 239 using DEO (de Boer *et al.*, 1988). They isolated 140 delation mutants defective in L-phenylalanine degradation among 140,000 colonies tested.

Treatment of R.rhodochrous PNKb1-Str^r_{20} with DEO (1 μl ml^{-1} culture) did not give a good kill curve, see Fig. 5.1. This mutagen might need longer incubation time. However, when the amount of DEO was doubled, cells were killed after 8 minutes incubation. This type of mutagen might not be suitable for selecting mutants from R.rhodochrous PNKb1-Str^r_{20}.

Incubation of R.rhodochrous PNKb1-Str^r_{20} with DEB (2 μl ml^{-1} culture) for 8.5 and 32 minutes gave a survival rate of 50% and 5% respectively (Fig. 5.1). Individual colonies obtained after DEB mutagenesis (yielding 50% and 95% survivors) were replica plated onto AMS plus succinate and nutrient agar plates. No auxotrophs or white colonies were isolated. The reason behind this is not known. However, the previous work showed that NTG is the best chemical mutagenesis with R.rhodochrous PNKb1.

Figure 5.1 Kill curve for R.rhodochrous PNKb1 using DEB & DEO



5.3 Isolation, Characterization and biochemical analysis of NTG-generated propane oxidation mutants of R.rhodochrous PNKb1-Str^r_{20}

5.3.1 alk^- mutants

Ashraf (1990) isolated alk^- mutants which still retained the ability to utilize all terminal and subterminal intermediates of propane oxidation as growth substrates except for acetol (Table 5.1). Ashraf suggested that "alk^- mutants are defective in the structural gene (s) (alk^-) or regulatory gene (s) (alkR) of an acetol/propane oxygenase system".

In this study, biochemical characterization was done with respect to SDS-PAGE analysis of soluble cell-free extracts of Alk^- mutants to compare the pattern and the synthesis of the propane-specific polypeptides with the wild-type. Also, the ability of Alk^- mutants to epoxidate propene to 1,2-epoxypropane was investigated.

Figure 5.2 shows SDS-PAGE analysis of soluble cell-free extracts from alk^- mutants after growth on propane and succinate [0.035% (w/v)] as growth supporting substrate. alk^- Mutants failed to synthesize all components of the propane specific polypeptides under propane-inducing conditions. This might suggest the involvement of a regulatory element [alkR, as previously reported by Ashraf

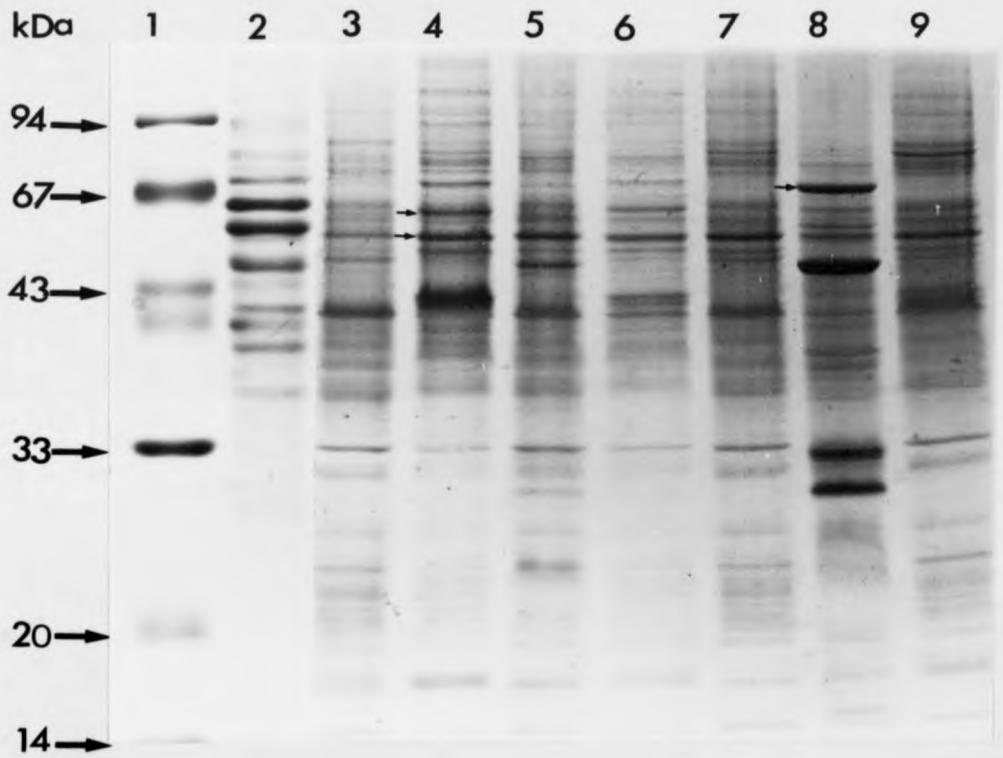
Figure 5.2 SDS-PAGE of cell-free extracts of *alk*⁻ mutants were grown on 0.035% (w/v) succinate under propane-inducing conditions.

Track	Contained
1	(Molecular weight markers)
2	W.T grown on propane
3	*alk* 3
4	*alk* 4
5	*alk* 10
6	*alk* 24
7	*alk* 25
8	*alk* 33
9	*alk* 50

100 μg protein in each track

WT = Wild-type

N.B All mutants were grown on propane + 0.035% (w/v) succinate


kDa
1
2
3
4
5
6
7
8
9
94
67
43
33
20
14

(1990)]. Nevertheless, all *alk*$^-$ mutants did synthesize at least one of the propane-specific polypeptides, the one of approximately 57 kDa, albeit at lower amounts when compared with the wild-type. *Alk* $_4$ synthesized two major propane-specific polypeptides of approximately 59 and 57 kDa, but lower amounts were observed when compared with the wild-type. *Alk* $_{33}$ also synthesized lower amounts of the propane-specific polypeptides of approximately 69 and 59 kDa. SDS-PAGE of soluble proteins from *alk*$^-$ mutants demonstrated that not all propane-specific polypeptides were synthesized under propane inducing conditions. This may be due to a mutation in a regulatory gene (s) (*alk*R) which lead to the failure of propane to induce the synthesis of these specific polypeptides. A similar phenomenon was observed with *alc*$^-$ mutants; see Ashraf (1990) for more details.

Alk$^-$ mutants were tested for their ability to epoxidate propene to 1,2-epoxypropane after growth on propane and 0.035% succinate as growth supporting substrate. None of the *alk*$^-$ mutants were able to carry out the epoxidation reaction. This demonstrates that an active propane oxygenase was not produced under propane-inducing conditions. It would be interesting to look for the epoxide formation by combining cell-free extracts of *Alk* 33 and *Alk* 4. Macham and Heydeman (1974) used similar techniques to identify the number of heptane monooxygenase components of the propane oxygenase system in *P.aeruginosa*.

5.3.2 *Ket*⁻ mutants

Two mutants which were defective in acetone metabolism (*ket*⁻) and still retained the ability to utilize all terminal and subterminal intermediates of propane oxidation as growth substrates were isolated (Table 5.2). The fact that *ket*⁻ mutants lost their ability to grow on propane or propan-2-ol suggested that acetone might be an intermediate in propane metabolism. These mutants were able to grow on both propan-1-ol and 1,2-propanediol but failed to grow on acetone or propan-2-ol, suggesting that propane might in these mutants be oxidized to propan-1-ol and/or 1,2-propanediol. Ashraf (1990) isolated two *ket*⁻ mutants which failed to grow on acetone and retained their ability to utilize propane, terminal and subterminal intermediates of propane oxidation (including propan-2-ol) as growth substrate, see Table 5.1. This led him to suggest that acetone is not an intermediate of propane oxidation and that propan-2-ol could be required a further hydroxylation to form 1,2-PDL and then followed by dehdration to produce acetol. The above results suggest that there might be tow different pathways occur for the oxidation of propan-2-ol; one being converting propan-2-ol to acetone and then to acetol and the other metabolizing propan-2-ol to acetol *via* 1,2-PDL.

Table 5.2 Characterization of Ket⁻ mutants

		Growth Substrates							
Class	No. of Mutants	Propane	Propan-1-ol	Propanal	Propanoate	1,2-Propanediol	Acetol	Propan-2-ol	Acetone
Ket⁻	2	-	+	+	+	+	+	-	-

+ = Growth - = No growth

NB Wild-type grows on all intermediates

5.3.3 *alcC*⁻ mutants

Four *alcC*⁻ mutants which had lost their ability to utilize 1,2-propanediol as growth substrate were isolated. Nevertheless, they still possessed the ability to utilize terminal and subterminal intermediates of propane oxidation (Table 5.3).

The inability of *alcC*⁻ mutants to grow on 1,2-propanediol and their ability to utilize propane and potential oxidation intermediates of terminal and subterminal pathways as growth substrates indicates that these tow pathways play a major role in the assimilation of propane. The ability of these mutants to utilize propane as a growth substrate indicate that propane might be oxidized subterminally to acetol *via* acetone and not 1,2-PDL. This pathway was also suggested due to the isolation of tow mutants that failed to grow on propane, propan-2-ol but retained their ability to utilize all terminal and subterminal intermediates of propane oxidation including 1,2-PDL (see section 5.3.2). An alternatively 1,2-PDL is probably not an intermediate of propane oxidation. However, this must be taken in caution. Previous results suggest that 1,2-propanediol might be an intermediate in the metabolism of propane for several reasons discussed in section 3.6. If it is possible to isolate mutants that are defective in the metabolism of acetol (*aol*⁻) which also failed to utilize both propane and

1,2-propanediol, this would provide evidence that 1,2-propanediol is an intermediate in the metabolism of propane.

5.4 Summary

All mutants discussed previously were derived from a R.rhodochrous PNKb1-Str^{r}_{20} using NTG-mutagenesis techniques. Neither auxotrophs nor white colonies were observed after DEO or DEB-mutagenesis, indicating that these two chemical mutagens are not effective with R.rhodochrous PNKb1.

Alk^{-} mutants failed to epoxidate propene to 1,2-epoxypropane in cell-free extracts of cells grown on succinate under propane inducing conditions implying that the propane oxygenase activity was not present. SDS-PAGE of soluble cell-free extracts from Alk^{-} mutants grown under propane inducing conditions showed the synthesis of one of the propane-specific polypeptides. However, Alk 4 synthesized lower amounts of two major propane-specific polypeptides, pointing to a mutation in the regulatory gene (s) (alkR) which lead to the failure of the propane molecule to induce the synthesis of these specific polypeptides.

Two ket^{-} mutants were isolated in this study which had lost the ability to utilize propane and propan-2-ol. This demonstrates that, in these mutants, acetone could be an intermediate of propane oxidation.

Table 5.3 Characterization of alc C⁻ mutants

		Growth Substrates							
Class	No. of Mutants	Propane	Propan-1-ol	Propanal	Propanoate	1,2-Propanediol	Acetol	Propan-2-ol	Acetone
alcC⁻	4	+	+	+	+	-	+	+	+

+ = Growth - = No growth

NB Wild-type grows on all intermediates

The ability of *alcC*$^-$ mutants to utilize propane, propan-1-ol and propan-2-ol as growth substrates suggests that propane is metabolized terminally *via* propan-1-ol and subterminally to acetol *via* propan-2-ol and acetone pathways.

CHAPTER 6

ELECTRON MICROSCOPIC STUDIES

6.1 Introduction

Whereas little is known about the ultrastructure of ethane, propane and butane-utilizing microorganisms, special attention have been given to methanotrophs. Two basic membrane arrangements have been observed in methanotrophs (type I and Type II, see section 1.2.2). These internal membrane structures have been postulated to be associated with methane assimilation. Nevertheless, obligate and facultative methylotrophs do not possess such internal membranes (Anthony, 1982).

McLee *et al.* (1972) observed unusual structures within the cytoplasm of n-butane-grown microorganisms which were absent when the same organisms were grown on glucose.

Kormendy and Wayman (1974) observed intracellular structures in two Arthrobacter sp. and in Candida utilis when cells were grown on either n-butane or 1-butanol but not glucose. These structures tend to be near the poles of the cells which consist of a large electron-dense bodies surrounded by a sphere-like electron-transparent area and connected to the cytoplasm by a fairly wide bridge. Kormendy and Wayman called these structures "oxisomes". They suggested that they are associated with hydrocarbon or alcohol oxidation.

A comparative study of the ultrastructures of three species of Rhodococcus rhodochrous and their surface structures grown on meat peptone agar (MPA) in the presence and absence

of propane was performed (Ivshina *et al.*, 1982). Scanning electron microscopic studies on propane-grown cells shows some circular protuberances at the poles of many cells. They proposed that these projections play a major role in ensuring contact between cells and fixing them in colonies. Propane-grown cells exhibit an increase in the number and size of the volutin granules. In addition, large electron-dense inclusion (EDI) surrounded by an electron-lucent zone were only observed in propane-grown cells. These EDI are very similar to the "oxisomes" described by Kormendy and Wayman (1974). Also, changes in the intracellular membrane structures were observed in terms of the formation of large tubular-vesicular structures in propane-grown cells. It has been suggested by several investigators that these structures increase the surface area of the cytoplasmic membrane and may be associated with hydrocarbons utilization (cited by Ivshina *et al.*, 1982).

6.2 Transmission electron microscopy study of propane-grown cells

Owing to the scant data on transmission electron microscopic studies of propane-utilizing bacteria, special consideration was given to the involvement of any unusual structures in propane-grown R.rhodochrous PNKb1.

The existence of the intracytoplasmic membrane in methane-grown Methylococcus capsulatus Bath was used as a comparative study between methanotrophs and propane-

utilizing bacteria. M.capsulatus Bath belongs to type X organisms. The membrane arrangement of M.capsulatus Bath is shown to consist of pairs of membranes aligned throughout the cells (Fig. 6.1).

Transmission electron micrographs in Fig. 6.2 a & b illustrated an ultra-thin sections of propane-grown Rhodococcus rhodochrous PNKb1. Large round dense inclusions (RDI) at the poles of the cells are shown in Fig. 6.2 a & b. These unusual structures could be the same as the electron dense inclusions (EDI) (Ivshina *et al.*, 1982) or what is known as oxisomes (Kormendy and Wayman, 1974) observed previously. These structures observed in R.rhodochrous PNKb1 were slightly different from those mentioned above. There was no transparente zone (TZ) surrounding these structures as found in propane-grown Rhodococcus rhodochrous (Ivshina *et al.*, 1982). These structures could represent sites of propane oxidation, and the absence of these structures in either succinate or pyruvate-grown R.rhodochrous PNKb1 should prove this suggestion. Figure 6.2a shows the division of growing cells with a large round dense inclusions (RDI) at each pole of the cells. The formation of a transverse septum in the middle of many cells is shown in Fig. 6.2a. The thin sectioning of propane-grown R.rhodochrous PNKb1 shows that the cells do not possess volutin granules, fatty inclusions or mesosomes.

Figure 6.1 Electron micrographs of ultra-thin sections of Methylococcus capsulatus Bath grown on methane showing a type I membrane system. Magnification x 15,000.

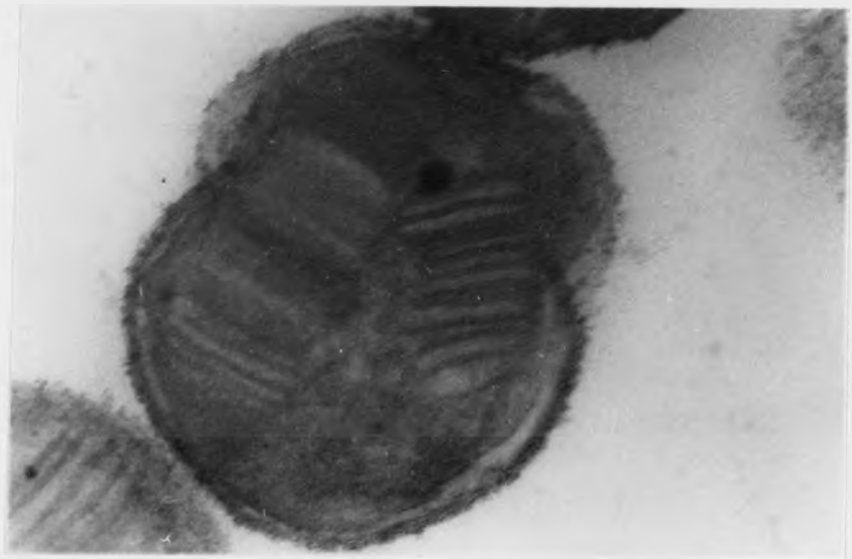
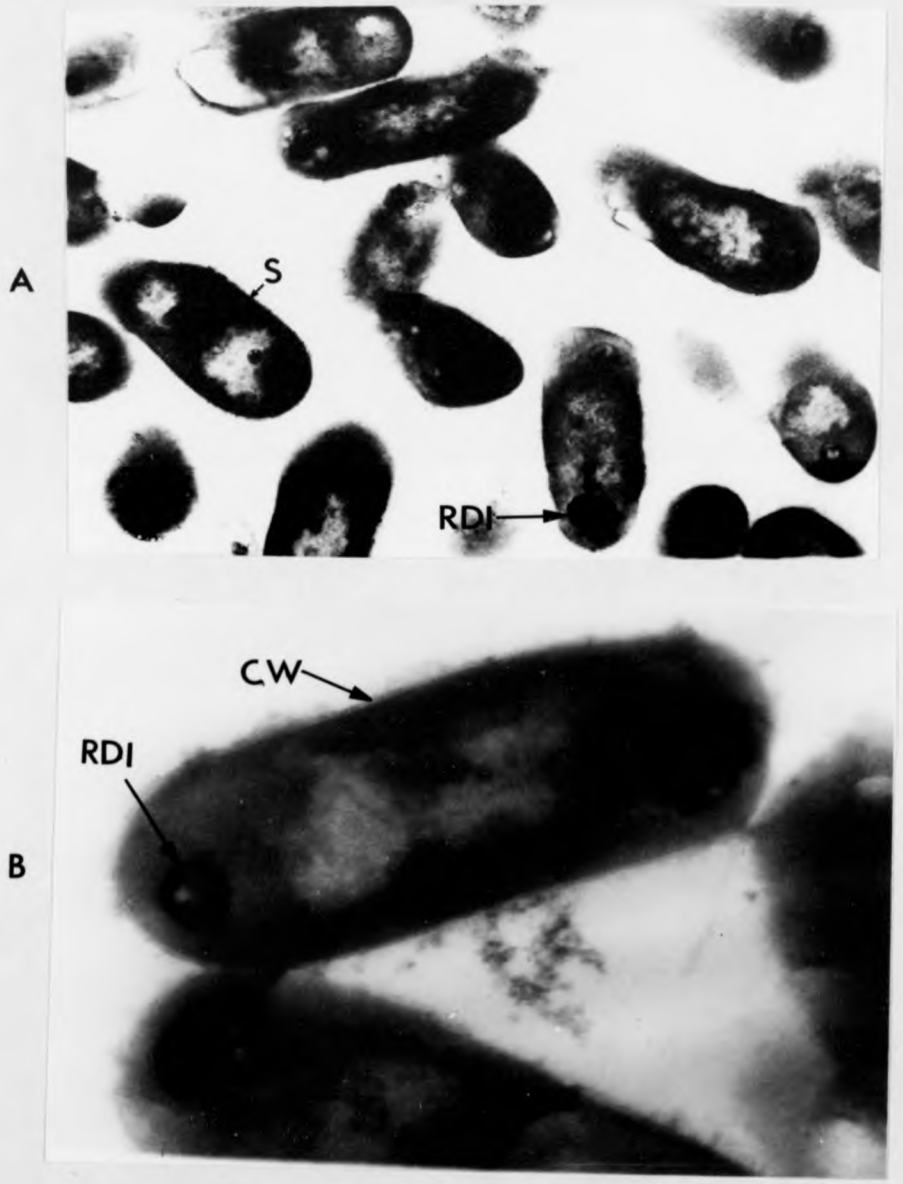


Figure 6.2 Electron micrographs of ultra-thin sections of Rhodococcus rhodochrous PNKb1 grown on propane. (a) Magnification x 10,000 (b) Magnification x 25,000. CW, cell wall; RDI, round dense inclusions; S, septum.



6.3 Summary

Transmission electron microscopic micrographs showed the involvement of unusual structures within the cells of propane-grown R.rhodochrous PNKb1. These structures were round dense inclusions (RDI) and found at the poles of the cells. Similar structures called electron dense inclusions (EDI) were observed in Rhodococcus rhodochrous after growth on meat-peptone agar (MPA) in the presence of propane (Ivshina *et al.*, 1982). They reported that these structures were absent when cells were grown on MPA, indicating that these structures could be associated with propane assimilation. Volutin granules, fatty inclusions or mesosomes were not observed in R.rhodochrous PNKb1.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

The metabolism of propane by R.rhodochrous PNKb1 has been previously investigated by Woods using different approaches such as growth substrate specificity, simultaneous adaptation studies in whole cells and enzyme activities in cell-free extracts (Woods, 1988). Ashraf (1990) continued the investigation on the pathway of propane oxidation using various techniques which were generally based on the isolation and characterization of NTG-generated mutants blocked in certain steps of terminal and subterminal intermediates of propane oxidation. The reason behind this strategy was to determine whether propane was metabolized *via* terminal or subterminal oxidation and to demonstrate the metabolic steps of the intermediates that are involved in the metabolism of propane. Antibodies were raised against the purified NAD^+-dependent secondary alcohol dehydrogenase from R.rhodochrous PNKb1 and used in Western-blot techniques to examine the induction of this enzyme in the wild-type organism after growth on propane and potential oxidation intermediates. Alc^- mutants grown under propane-inducing conditions were also tested for the induction of this enzyme. In addition, genetic approaches were unsuccessful in trying to develop a transformation system for R.rhodochrous PNKb1. The above different approaches confirmed that terminal and subterminal oxidation are involved in the metabolism of propane. However, none of these approaches gave a clear answer as to whether or not 1,2-propanediol is involved in the metabolism of propane.

The aims of this study were focused to testify the route of the subterminal oxidation and to investigate the pathway of 1,2-propanediol metabolism and its link with propane metabolism, using NTG-generated mutants blocked at specific steps in propane and 1,2-propanediol metabolism. In addition, a comparative study between propane and acetol oxygenase(s) was carried out which lead to partial purification of acetol monooxygenase. Transmission electron microscopic studies were finally done to investigate the involvement of any unusual structures within the cytoplasm of propane-grown cells.

SDS-PAGE has demonstrated that acetone-grown cells synthesized the propane-specific polypeptides found in propane-grown cells. Acetone-grown cells also synthesized the NAD^+-dependent secondary alcohol dehydrogenase in low amounts when compared with propane and propan-2-ol-grown cells. These results suggest a relationship between the metabolism of propane and acetone. The accumulation of acetone during growth on propan-2-ol indicated that propan-2-ol could be metabolised 1,2-PDL and then to acetol. This pathway was previously suggested by Ashraf (Ashraf, 1990). Stephens (1983) reported that the accumulation of a proposed intermediate in the metabolism of a substrate should not normally occur unless if it was rapidly oxidised by the organism. In the case of R.rhodochrous PNKb1, acetone was only a transitory product which was further metabolised only when the medium was lacking in the substrate. Also acetnoe

was oxidised by propane-grown cells (Ashraf, 1990). This suggests that the major route of propan-2-ol oxidation is through acetone and acetol pathways (Fig. 7.1). Two *ket*$^-$ mutants were found to be defective in propane and propan-2-ol metabolism. This suggests that acetone could be an intermediate of propane oxidation. However, Ashraf (1990) isolated two mutants defective in acetone metabolism (ket$^-$) which still retained the ability to utilize propane, terminal intermediates and subterminal intermediates (including propan-2-ol) as growth substrate. This lead him to suggest that acetone is not an intermediate of propane oxidation. A general suggestion from the above results can be concluded that subterminal oxidation proceeds through acetol *via* tow different pathways; propan-2-ol could be either metabolised to acetone or hydroxylated to 1,2-PDL and then dehydrated to acetol.

The isolation of *alcC*$^-$ mutants demonstrate that terminal and subterminal oxidations are the major route in the metabolism of propane. It would be interesting to test whether *alcC*$^-$ mutants synthesize the propane-specific polypeptides under propane inducing conditions. The isolation of *alcC*$^-$ and *aol*$^-$ mutants would indeed infer the involvement of propane dioxygenase in the metabolism of propane. SDS-PAGE analysis showed the induction of the propane-specific polypeptides after growth on 1,2-propanediol, indicating that there is a relationship between the metabolism of propane and 1,2-propanediol in *R.rhodochrous* PNKb1. Western-blot analysis of

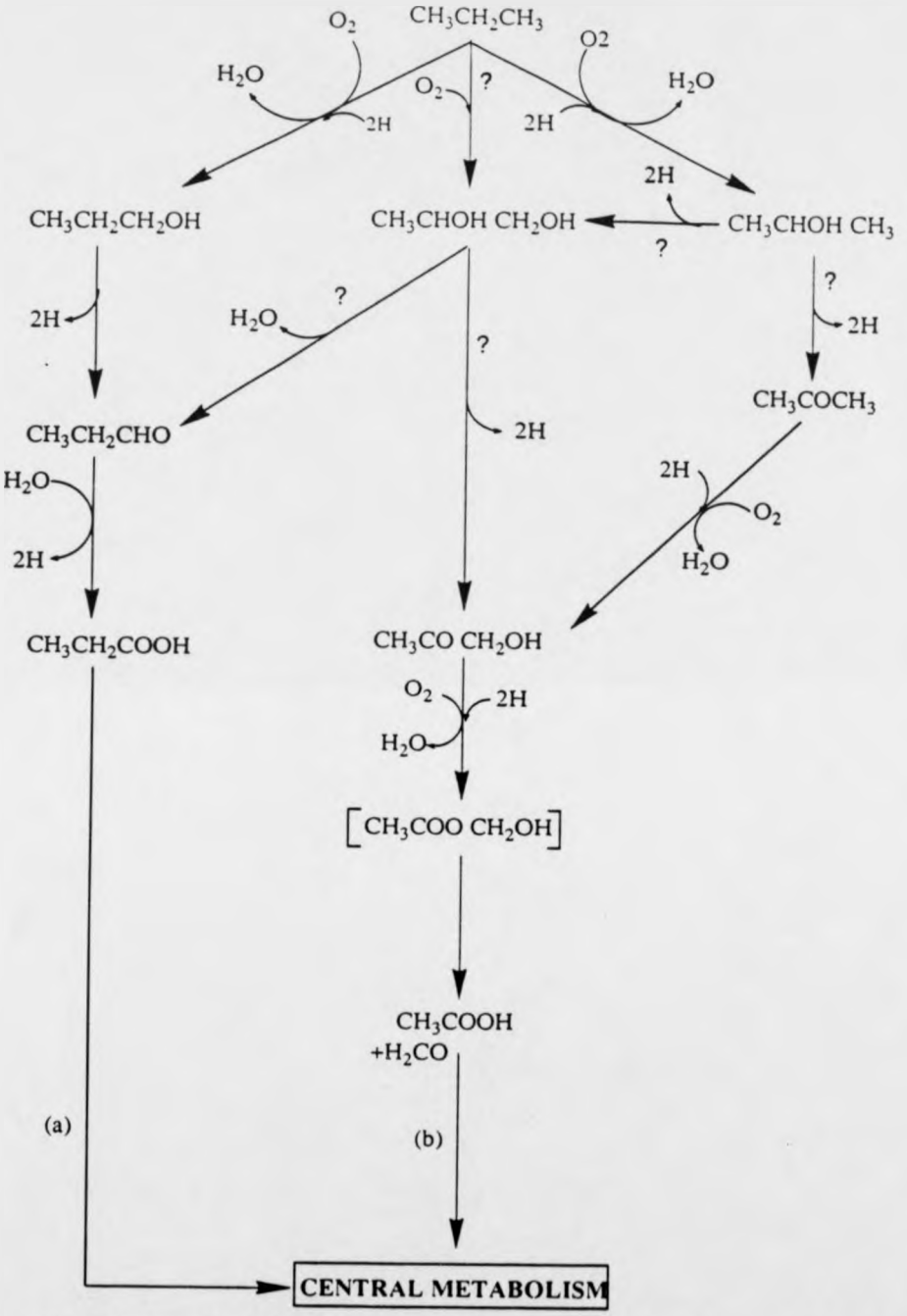


Figure 7.1 Proposed propane oxidation pathway by Rhodococcus rhodochrous PNKb1. (a) Terminal oxidation via propanoate; (b) sub-terminal oxidation via acetol and hydroxymethyl acetate.

cell-free extracts of 1,2-propanediol-grown cells also shows the synthesis of the secondary alcohol dehydrogenase at a lower amount from that found in propane-grown cells. Simultaneous adaptation studies showed that propane is oxidized by propane, acetol and 1,2-propanediol-grown cells (Ashraf, 1990). Tis suggests a relationship between the metabolism of these compounds. The transient accumulation of acetol from 1,2-propanediol-grown cells suggests that acetol is an intermediate of 1,2-propanediol metabolism. This could also mean that 1,2-propanediol might be oxidized to propanoate via propanal (see Fig. 7.1). Studies carried out by de Bont *et al.*(1982) on Nocardia sp. suggested that 1,2-propanediol was metabolized to succinate via propanoate pathway. Elevated levels of diol dehydratase was detected from 1,2-propanediol-grown Nocardia sp. It would be interesting to detect the activity of diol dehydratase in R.rhodochrous PNKb1 after growth on propane and 1,2-propanediol. The above results suggest that propane might be metabolized to propan-2-ol, 1,2-propanediol and then to acetol.

This study has shown that methyl acetate is not an intermediate in the metabolism of propane. This was confirmed by the excretion of methanol from methyl acetate-grown cells but not propane, acetone or propan-2-ol-grown cells (Fig. 3.1). Forthermore, methanol was not further metabolized or oxidized in R.rhodochrous PNKb1. SDS-PAGE of soluble proteins from methyl acetate-grown cells confermed that these cells did not synthesize the propane-specific

polypeptides. Also, there is a great difference between the protein profiles from cell-free extracts of propane and methyl acetate-grown cells, suggesting that a different and separate pathway is involved in the metabolism of methyl acetate. Taylor *et al.* (1980) failed to demonstrate the involvement of methyl acetate in the metabolism of propane-grown M.vaccae JOB5.

SDS-PAGE analysis of cell-free extracts from alk$^-$ mutants grown under propane inducing condition shows the synthesis of low amounts of at least one of the propane-specific polypeptides. Tow alk$^-$ mutant synthesized two major propane-specific polypeptides, suggesting that alk$^-$ mutants are defective in a regulatory gene(s) (alkR) of propane/acetol oxygenase system. A similar phenomenon was observed by Ashraf (Ashraf, 1990) with alcB 12 which synthesized only two major propane-specific polypeptides while alcB 8 failed to synthesized propane-specific polypeptides as well as NAD^+-dependent secondary alcohol dehydrogenase.

Elevated levels of NAD^+-dependent secondary alcohol dehydrogenase activity were observed in cell-free extracts of propane and propan-2-ol-grown R.rhodochrous PNKb1, while lower levels of primary alcohol dehydrogenase activity were noted in soluble extracts of propan-1-ol, propan-2-ol and 1,2-propanediol-grown cells. These results, along with low rates of diol alcohol dehydrogenase activity in cell-free extracts of propane and propan-2-ol-grown cells, suggest

that there is more than one alcohol dehydrogenase in the metabolism of propane.

Propan-2-ol was the only alcohol formed from propane-grown cells in the presence of ammonium chloride or phosphate, indicating that subterminal oxidation is the major route of propane metabolism. This phenomenon was also observed in other propane-utilizing bacteria. Strain E12 excreted both propan-1-ol and propan-2-ol after growth on propane in the presence of ammonium chloride as the nitrogen source. (Stephens, 1986). However, no alcohols were detected when strain E12 was grown on propane in the presence of KNO_3 in place of NH_4Cl as the nitrogen source.

The ability of R.rhodochrous PNKb1 to grow on or oxidize substituted propane compounds (e.g. 1-chloropropane, 2-chloropropane and 1,2 di-chloropropane) were tested in order to confirm whether a propane mono- or di-oxygenase was present in R.rhodochrous PNKb1. Unfortunately, none of the above chloropropane compounds were utilized or oxidized by R.rhodochrous PNKb1. These compounds were shown to be inhibiting the propane oxidation due to the observation that PNKb1 grows on 0.1% succinate (w/v) in the presence of chloropropane compounds while no growth was noted when cells were grown on the above propane analogues in the presence of propane. SDS-PAGE analysis of cell-free extracts of R.rhodochrous PNKb1 grown on 0.035% succinate (w/v) under propane-inducing condition in the presence of substituted propane compounds shows the synthesize of the propane-

specific polypeptides. However, cells grown on 0.035% succinate (w/v) undr propane-inducing condition in the presence of 2-chloropropane did not synthesized a 69 kDa specific polypeptide. The reason behind this is not known.

Due to the instability of propane oxygenase activity in cell-free extracts, formation of 1,2-epoxypropane from propene was used as an indicator for propane oxygenase activity. Nevertheless, the epoxide formation was inhibited by the additional of propane during the conversion of propene to 1,2-epoxypropane. V_{max} values of propene in the absence or presence of propane as inhibitor were approximately the same, implying that propane competes with propene for the same active site on propane oxygenase. Thus, the epoxide formation is indeed catalysed by the propane oxygenase.

Studies with propane, 1,2-propanediol, acetone and acetol-grown cells showed that only propane and acetol-grown cells had the ability to epoxidate propene to 1,2-epoxypropane. Interestingly, acetol, acetone and 1,2-propanediol-grown cells were adapted to oxidized propane. This may suggests that there are two different oxygenase activities in the metabolism of propane; a common propane/acetol oxygenase which can carry out the epoxidation reaction and an oxygenase induced after growth on 1,2-propanediol and acetone which cannot perform these transformations. However, acetone-grown cells contained additional specific polypeptides of approximately 92 and 84 kDa while additional

polypeptide of 36 kDa was observed with 1,2-PDL-grown cells. These polypeptides might be required specifically for the metabolism of acetone and 1,2-propanediol respectively. Alternatively, propene might be metabolized to allyl alcohol or acrylic acid. A common propane/acetol oxygenase system has been suggested due to the fact that rates of epoxide formation in cell-free extracts of propane and acetol-grown cells were relatively close (80 and 53 nmol 1,2-epoxypropane formed min^{-1} mg protein $cells^{-1}$ respectively). The rates of epoxide formation were measured in whole cells of propane and acetol-grown cells in the presence of various potential inhibitors. Both propane and acetol-grown cells were affected by the same inhibitors. Yet 8-hydroxyquinoline and 2-mercaptoethanol show a marked effect with acetol-grown cells but only a slight effect with propane-grown cells. It has been interestingly found that the activity of acetol oxygenase was quite stable at $0^{o}C$. However, propane oxygenase activity is extremely unstable. These observations lead to partial purification of acetol oxygenase from acetol-grown cells. Acetol oxygenase activity was only detected in cell-free extracts of acetol-grown cells. No NAD^{+} or $NADP^{+}$-dependent acetol dehydrogenase activity was detected in the soluble proteins of acetol-grown cells. Acetol monooxygenase has an optimum pH of 7 and an optimum temperature of $40^{o}C$. It would be interesting to continue the purification of this enzyme to homogeneity. Thus the characterization of this enzyme would give clear evidence for the existence of at least two oxygenase(s) systems in R.rhodochrous PNKb1.

Due to the scant data on the ultrastructure of propane utilizers, special attention was given to the intracellular structures of propane-grown R.rhodochrous PNKb1. Transmission electron microscopy has demonstrated the involvement of unusual structures within the cytoplasm of propane-grown cells. These structures contained large round dense inclusions (RDI) at the poles of the cells. Similar structures which are known as electron-dense inclusions (EDI) were described in Rhodococcus rhodochrous after growth on meat-peptone agar (MPA) in the presence of propane but not on MPA-grown cells (Ivshina *et al.*, 1982). The absence of these structures when cells were grown on MPA suggests that these structures are associated with propane assimilation. It would be interesting to test whether or not succinate-grown R.rhodochrous PNKb1 form the RDI structures. The presence of any unusual structures within the cells of propane-utilizers after growth on propane can be also compared with EDI or RDI that are found in Rhodococcus rhodochrous (Ivshina *et al.*, 1982) and R.rhodochrous PNKb1 respectively.

7.2 Future studies

Results from this thesis provides the basis for future studies on the metabolism of propane by R.rhodochrous PNKb1. For instance, the purification of acetol monooxygenase could be continued to homogeneity. Thereafter, antibodies could be raise against this enzyme and use in Western-blot techniques

to define the protein responsible for the oxidation of acetol and other intermediates. It would be also interesting to test the synthesize of this enzyme in *alk*$^-$ mutants grown on succinate under propane inducing conditions. *Alc*C$^-$ mutants require more investigation in terms of analysing the induction of the propane-specific polypeptides and the synthesize of NAD^+-dependent secondary alcohol dehydrogenase. The isolation of *aol*$^-$ and *alc*C$^-$ mutants is also required to test whether or not they are defective in the metabolism of propane (*alk*$^-$). Detection of diol dehydratase activity in cell-free extracts of propane and 1,2-propanediol-grown cells could also be tested. Attempts could be also made to purify "1,2-propanediol dehydrogenase" from propane-grown cells. This would help to classify the role of 1,2-PDL in the oxidation of propane.

The biochemical analysis of *ket*$^-$ mutants could be also carried out in terms of the induction of the propane-specific polypeptides and their ability to form 1,2-epoxypropane from propene when cells are grown under propane inducing conditions.

SDS-PAGE analysis of any *alk*$^-$ revertants would mutants demonstrate which propane-specific polypeptides were absent. Also, *in vitro* complementation studies, by combining cell-free extracts of specific *alk*$^-$ mutants could be use to test for the formation of 1,2-epoxypropane from propene by mutants grown under growth supporting propane oxygenase inducing conditions. Similar techniques were used to

identify the number of heptane monooxygenase components in P.aeruginosa (Macham and Heydeman, 1974). This would strongly define the components of the propane oxygenase system.

Addition of ammonium chloride, which had little effect on alcohol oxidation, resulted in the accumulation of propan-2-ol from propane-grown cells. In contrast, no alcohols were detected when cells were grown on propane with 50 mM EDTA in place of ammonium chloride. The concentration of EDTA used in this study possibly inhibited growth. It would be interesting to use a low concentration which might result in the accumulation of one or more alcohols.

Finally, ultrastructure studies of R.rhodochrous PNKb1 after growth on succinate should be made and compared with propane-grown cells in terms of involvement of RDI. This study could be a starting point for further studies on other propane-utilizing bacteria.

7.3 Synopsis

As results have been discussed at the end of each chapter, this synopsis will briefly summarize the significant findings of the results presented in this thesis.

(1) Acetone was suggested to be an intermediate in the subterminal oxidation of propane.
(2) Accumulation of propan-2-ol from propane-grown cells in

the presence of NH_4Cl suggested that the major route of propane metabolism proceeds *via* the oxidation of the subterminal carbon atom.

(3) Propane oxidation does not proceed *via* methyl acetate.

(4) Biochemical analysis of *alk*$^-$ mutants shows the absence of the biotransformation of propene to 1,2-epoxypropane. Also, SDS-PAGE analysis demonstrated the synthesis of at least one component of the propane-specific polypeptides.

(5) There is more than one alcohol dehydrogenase involved in the metabolism of propane.

(6) Propane is a competitive inhibitor of propene oxidation, acting at the same active site on propane oxygenase, indicating that epoxide formation is catalysed by the propane oxygenase system.

(7) Inhibitor studies and the partial purification of acetol monooxygenase demonstrates that there might be meaner differences between propane oxygenase and acetol monooxygenase.

(8) Transmission electron microscopic studies shows the presence of unusual structures (RDI) at the poles of *R.rhodochrous* PNKb1 after growth on propane.

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